Proceedings
of the
Society
for
Experimental Biology and Medicine

VOLUME 61 MARCH, 1946 NUMBER 3

SECTION MEETINGS

DISTRICT OF COLUMBIA	George Washington University December 6, 1945 February 7, 1946
NEW YORK	New York Academy of Medicine February 20, 1946
SOUTHWESTERN	University of Oklahoma Medical School December 27, 1945
WESTERN NEW YORK	University of Rochester February 16, 1946

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Influence of Thiourea on Organ Weights of Rats as Related to Food Intake.

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Thyroid hypertrophy is invariably observed following the administration of antithyroid drugs but the influence on other body organs is not definitely established.[1] Since it is known that thiourea or thiouracil will reduce food intake[2-6] and since inanition alone will alter organ weights, the present investigation was undertaken to determine the influence of reduced food intake alone on organ weights. Therefore, organ weights of thiourea-fed, pair-fed and ad-libitum-fed rats were compared.

Male rats of the Long-Evans strain, ranging from 139 to 162 days in age, were used. These animals were kept in metabolism cages for the measurement of daily food intake. Thiourea was fed as half of one percent of the stock diet for a period of 20 to 27 days. At autopsy, the fresh weight of the pituitary, adrenals, thyroid, seminal vesicles (including the coagulating gland and any contained fluid), kidneys and liver was recorded. The anterior pituitary was assayed for gonadotropic hormone content in the same manner as reported previously.[3]

Average food consumption per rat during the first 20 days totaled 258 g for the thiourea-fed rats as compared with 340 g for normal rats eating ad libitum. Furthermore, the thiourea-fed rats exhibited a body weight

1 Riker, W. F., and Wescoe, W. C., *Am. J. Med. Sc.*, 1945, **210**, 665.

2 Williams, R. H., Weinglass, A. R., Bissell, G. W., and Peters, J. B., *Endocrinology*, 1944, **34**, 317.

3 Leathem, J. H., *Endocrinology*, 1945, **36**, 98.

4 Astwood, E. B., Sullivan, J., Bissell, A., and Tyslowitz, R., *Endocrinology*, 1943, **32**, 210.

5 Hughes, A. M., *Endocrinology*, 1944, **34**, 69.

6 Gordon, A. S., Goldsmith, E. D., Charipper, H. A., *Fed. Proc.*, 1945, **4**, 25.

TABLE I.
Influence of Thiourea on Organ Weights of Rats.

Teatment (No. of rats)	Avg organ wt g		Avg organ wt g per 100 g body wt		
	Thiourea fed (20)	Normal pair-fed (20)	Thiourea fed (20)	Normal pair-fed (20)	Normal ad. lib. fed (20)
Pituitary	0.009	0.009	0.004	0.003	0.003
Adrenals	0.025	0.030	0.010	0.011	0.010
Thyroid	0.036	0.022	0.014	0.008	0.008
Testes	2.899	2.856	1.126	1.020	1.063
Seminal vesicles	0.694	1.082	0.267	0.382	0.377
Kidney	2.533	2.545	0.977	0.919	0.891
Liver	10.082	10.525	4.057	3.772	3.984

TABLE II.
Gonadotrophic Potency of Rat Pituitaries.

Donors		Recipients			
				Avg organ wt — mg	
Treatment	Amt A.P.	No. of rats	Avg body wt	Ovaries	Uteri
	mg		g		
—	—	10	55.1	13.4 ± 1.0*	24.7 ± 1.4
Thiourea	8.9	13	59.4	91.0 ± 7.5	72.1 ± 7.2
None—pair fed	8.5	13	57.2	94.0 ± 6.5	68.7 ± 4.2

* Mean deviation of mean $= \epsilon = \sqrt{\frac{\Sigma d^2}{n(n-1)}}$

loss averaging 46 g whereas the normal rats gained 17 g. The pair-fed normal controls lost only 19 g in weight although food intake was restricted to that of thiourea-fed rats.

Examination of organ weights revealed the anticipated thyroid hypertrophy but the weights of the adrenals, testes, kidney and liver were not affected. The pituitaries were slightly heavier following thiourea feeding but the increase was not significant. Furthermore, the degree of food restriction imposed on the rats pair-fed with the thiourea-fed rats was not sufficient to alter the normal organ weight/body weight ratio (Table I). Other investigators have also indicated that the above organs do not change in weight in animals receiving antithyroid drugs.[2,3,7-9] However, Baumann and Marine[10] have reported a definite decrease in adrenal weight due to cortical atrophy in the presence of slight medullary hypertrophy whereas Kennedy and Purves[11] observed adrenal hypertrophy. An increase in pituitary, kidney and liver size has also been reported to occur when thiourea or thiouracil is administered.[8] Although our data do not reveal an influence of thiourea on most organs over the 20-day period we have consistently obtained a reduction in seminal vesicle weight (Table I). It was of interest to find that gonadotropic hormone of the anterior hypophysis of thiourea-fed and pair-fed rats did not differ although the decrease in seminal vesicle weight suggested a decrease in release of luteinizing hormone (Table II).

Summary. The reduction in food intake caused by the addition of a half of one percent of thiourea to the diet for 20 days did not influence organ weight/body weight ratios in spite of a body weight loss. In addition to thyroid hypertrophy, a decrease in

[7] Richter, C. P., and Clisby, K. H., *Arch. Path.*, 1942, **33**, 46.

[8] Leblond, C. P., and Hoff, H. E., *Endocrinology*, 1944, **35**, 229.

[9] Goldsmith, E. D., Gordon, A. S., and Charipper, H. A., *Endocrinology*, 1945, **36**, 364.

[10] Baumann, E. J., and Marine, D., *Endocrinology*, 1945, **36**, 400.

[11] Kennedy, J. H., and Purves, H. D., *Brit. J. Exp. Path.*, 1941, **22**, 241.

seminal vesicle weight was evident after feeding thiourea, although the gonadotropic hormone content of the pituitary of thiourea-fed and pair-fed rats did not differ. Pituitary, testis, adrenal, kidney and liver weights were not significantly changed.

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Temperature Coefficients of Hemolysis of a Few Types of Nucleated Erythrocytes.*

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Jacobs *et al.*[1] have presented a series of data giving temperature coefficients for the hemolysis of various types of mammalian erythrocytes in non-electrolytes. These data

TABLE I.
Temperature Coefficients for Hemolysis in Isosmotic Solutions.

Type of blood	Penetrating substance	Time in seconds for 80% hemolysis 10°	20°	30°	40°	50°	Mean Q_{10} 10°-50° (except when enclosed in parentheses)
Turtle	H_2O	6.4	5.5	4.6	4.0	2.8	1.23
	Urea	9.9	8.8	8.7	7.2	5.3	1.17
	Ethylene glycol	107.0	41.4	22.2	11.6	6.7	2.00
	Methyl alcohol	7.4	8.0	6.4	5.2	3.8	(1.28)
	Ethyl "	8.5	7.0	5.3	5.0	3.9	1.22
	Propyl "	9.7	9.6	7.8	6.1	4.8	(1.26)
Chicken	H_2O	38.2	21.3	13.6	8.5	5.0	1.66
	Urea	879.0	476.0	322.0	134.0	71.4	1.87
	Ethylene glycol	85.8	42.2	23.6	12.2	7.2	1.86
	Glycerol		1456.0	455.0	150.0	81.5	(2.61)
	Methyl alcohol	34.8	20.8	13.1	8.0	4.6	1.66
	Ethyl "	35.7	20.5	14.1	7.4	4.4	1.69
	Propyl "	37.4	21.2	13.5	8.4	4.4	1.70
Monk Fish	H_2O	116.0	73.7	62.3	48.5	6.5	2.06
	Urea	1623.0	551.0	299.0	178.0	75.3	2.16
	Glycerol	2625.0	905.0	342.0	143.0	61.9	2.55
	Methyl alcohol	449.0	346.0	103.0	23.2	10.9	2.53
	Propyl "	171.0	107.0	79.2	11.3	5.2	2.39
Dog Fish	H_2O	50.0	24.8	15.4	8.4	5.1	1.77
	Urea	1030.0	518.0	264.0	128.0	65.7	1.99
	Ethylene glycol	117.0	55.3	25.8	13.8	8.3	1.94
	Glycerol	2515.0	1114.0	724.0	293.0	141.0	2.05
	Methyl alcohol	41.7	27.8	14.4	7.9	5.1	1.69
	Ethyl "	43.8	27.6	13.7	7.6	5.1	1.71
	Propyl "	50.9	29.3	15.3	7.0	4.2	1.87
Briar Skate	H_2O	65.4	32.8	19.7	10.7	4.7	1.93
	Urea	1132.0	309.0	163.0	78.2	16.8	2.87
	Ethylene glycol	169.0	47.6	33.6	14.0	7.6	2.17
	Glycerol	2593.0	599.0	287.0	138.0	44.3	2.77
	Methyl alcohol	45.2	19.8	14.0	85.0	4.0	1.83
	Ethyl "	57.5	25.0	14.2	9.1	3.1	2.08
	Propyl "	57.7	28.9	9.9	4.9	1.8	2.38
Barn door Skate	H_2O	113.0	46.6	24.7	14.3	6.7	2.03
	Urea	753.0	393.0	142.0	69.4	27.5	2.29
	Ethylene glycol	183.0	75.7	42.7	27.1	11.2	2.01
	Glycerol	2756.0	883.0	315.0	194.0	80.2	2.42
	Methyl alcohol	81.9	50.1	25.3	13.9	5.6	1.96
	Ethyl "	86.6	42.5	22.1	12.2	5.1	2.03
	Propyl "	103.0	56.8	25.0	9.7	3.8	2.28

Avg values based on 1,421 individual observations.

* The experiments herein reported were performed in the Zoology Department, Rhode Island State College, Kingston, R. I.

[1] Jacobs, M. H., Glassman, H. N., and Parpart,

have recently been utilized in an attempt to relate species differences in permeability to possible chemical differences in the cell membrane (Dziemian[2] and Ballentine.[3])

Because of their possible use in future studies, and for comparative purposes, the data in Table I are presented giving temperature coefficients of hemolysis of nucleated erythrocytes of several species.

The technic employed in these experiments was essentially that used by Jacobs *et al.*[1] In general, the temperature coefficients of hemolysis for these nucleated erythrocytes are of the same order of magnitude as those obtained using mammalian red cells. These data are too fragmentary to justify a detailed analysis. However, certain comparisons can well be made with data described by Jacobs and Glassman.[4] These authors point out that in fishes (elasmobranchs and teleosts) the permeability to ethylene glycol is high, in birds the permeability to ethylene glycol and glycerol is very great and nearly equal, and much less to urea, except in the duck and chicken, and permeability to urea is great in reptiles. The present data are in complete agreement. Ethylene glycol permeability is high in the four fishes studied, urea and glycerol permeability is high in the chicken, and the turtle exhibits a very high permeability to urea.

Arthur K., *J. Cell. and Comp. Physiol.*, 1935, **7**, 197.

[2] Dziemian, Arthur J., *J. Cell. and Comp. Physiol.*, 1939, **14**, 103.

[3] Ballentine, Robert, *J. Cell. and Comp. Physiol.*, 1944, **23**, 21.

[4] Jacobs, M. H., and Glassman, H. N., *Biol. Bull.*, 1937, **73**, 387.

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Isolation of St. Louis Encephalitis Virus from a Fatal Human Case in California.*†

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Hammon and Reeves[1] have recently reported recovery of the St. Louis encephalitis virus from naturally infected mosquitoes (*Aedes dorsalis* Meigen) caught in Kern County, California, in 1944, the first isolation of this virus from any source in this state.

The present report describes the isolation of the St. Louis virus from a fatal human case in California. This is the first time the virus has been isolated from any vertebrate host outside the endemic St. Louis area, and furnishes additional, more direct, evidence to that obtained from serologic studies,[2-4] of the activity of this virus in California.

The patient was an itinerant agricultural worker who, during the past year, had resided in Kern County in the neighborhood

* The work on which this paper is based was conducted with the support and under the auspices of the International Health Division of The Rockefeller Foundation in cooperation with the California State Department of Public Health.

† The author is indebted to Dr. John J. Sippy, District Health Officer of the San Joaquin Local Health District, for clinical and epidemiologic data, and to Dr. Elmer W. Smith, Pathologist to the San Joaquin General Hospital, for information on the clinical and post-mortem findings and for furnishing pathologic material for examination.

[1] Hammon, W. McD., and Reeves, W. C., *Am. J. Pub. Health*, 1945, **35**, 994.

[2] Wynns, H. L., and Hawley, C. J., *Am. J. Pub. Health*, 1939, **29**, 781.

[3] Howitt, B. F., *Am. J. Pub. Health*, 1939, **29**, 1083; ibid., 1942, **32**, 503.

[4] Hammon, W. McD., Reeves, W. C., and Galindo, P., *Am. J. Hygiene*, 1945, **42**, 299.

of Bakersfield and Arvin. He arrived in Stockton (San Joaquin County) on September 4 or 5, 1945, and lived at an auto trailer camp about 6 miles east of the city. On the morning of September 11 he was observed to be irrational and to walk with a stumbling gait, and was hospitalized with a diagnosis of acute encephalitis of undetermined etiology. Examination of the spinal fluid on admission showed the following: 10 red cells, 20 white cells, Pandy's reaction +, Gram stain for organisms negative, sugar 81.4 mg per 100 cc, chloride 720 mg per 100 cc, Kolmer's test 4 +, colloidal gold curve 0-0-1-2-1-0-0-0-0. Blood examination on admission showed: hemoglobin 83 percent, white blood cells 20,900, polymorphonuclears 76, lymphocytes 14, mononuclears 10, stabs 5, Kolmer and Kline tests both 4 +. The temperature at the time of admission was 107.4°; under treatment with medication and icecaps it dropped to 103° by the 3rd day, then rose to 105.3° at the time of death on the 4th hospital day. On post-mortem examination there was found some passive congestion of the lungs, a few scars in the liver, and an acute congestion and severe edema of the brain; the rest of the organs showed nothing unusual.

A 20 percent suspension of brain tissue in 10 percent normal rabbit serum broth was prepared and spun at 1500 r.p.m. for 15 minutes in an angle centrifuge. The supernatant fluid was inoculated intracerebrally (0.1 ml) into 2 guinea pigs, intraperitoneally (0.03 ml) into 8 mice 14 days of age, and by the combined intracerebral (0.03 ml) and intraperitoneal (0.1 ml) routes into 8 mice 20 days of age. Neither guinea pig showed evidence of infection during the one month observation period. Of the 14-day-old mice, one was found dead on the 8th day and one on the 9th day; 2 were ill on the 9th day and were found dead on the 10th day; and 4 survived and were discarded on the 21st day. Of the 20-day-old mice, one was found dead on the 5th day and the remaining 7 were obviously ill on the 7th day, showing ruffled fur, hunching, tremors, and ataxia. Four of these animals were killed, and their brains were pooled and passaged by combined intracerebral-intraperitoneal inoculation into 14- to 15-day-old mice; the remaining 3 animals were killed on the 12th day and their brains similarly inoculated in 15-day-old mice. In both cases the passage mice showed ruffling of the fur, convulsions, tremors, ataxia, and paralyses of hind or fore limbs after an incubation period of 4 to 6 days.

Aerobic and anaerobic bacteriologic cultures of infected brains were either sterile or occasionally showed the presence of a few saprophytic bacteria. Since Berkefeld N and W, and Seitz EK filtrates were bacteriologically sterile yet highly pathogenic for mice, the agent was regarded as a virus. For convenience, it has been designated the Winkler strain.

In an attempt to identify the agent, guinea pigs and hamsters were inoculated intracerebrally (0.1 ml), intraperitoneally (0.5 ml), or by both routes combined, with a 1 percent suspension of brains from the 3rd mouse passage. Three animals were inoculated by each route; all survived without showing any evidence of infection during a 3-week observation period. The lack of pathogenicity for guinea pigs was considered possibly to rule out several viruses, and the failure to produce apparent infection in hamsters to rule out others, including the St. Louis virus, to which this species has been reported as susceptible.[5,6] Nevertheless, on the assumption that newly isolated strains of a virus might fail to produce obvious infection in species normally susceptible to highly passaged laboratory strains of the same virus, neutralization tests were done using the Winkler virus and rabbit or guinea pig immune sera to the Eastern equine encephalomyelitis, Western equine encephalomyelitis, lymphocytic choriomeningitis, Japanese B, St. Louis (Hubbard strain) and Winkler viruses, and to an unidentified virus isolated from mosquitoes of California by Hammon

[5] Broun, G. O., Muether, R. O., Mezera, R. A., and LeGier, M., PROC. SOC. EXP. BIOL. AND MED., 1941, **46**, 601.

[6] Lennette, E. H., PROC. SOC. EXP. BIOL. AND MED., 1941, **47**, 178.

TABLE I.
Effect of Age of Animal and Route of Inoculation on the Isolation of St. Louis Encephalitis Virus in Mice.

Route of Inoculation	Age of mice: 14 days Mortality ratio*	14 days % Mort.	14 days A.S.T.†	21-28 days Mort. ratio	21-28 days % Mort.	21-28 days A.S.T.	6-7 weeks Mort. ratio	6-7 weeks % Mort.	6-7 weeks A.S.T.
			days			days			days
I.P.	20/24	83	11.0	5/27	18	18.9	3/18	17	19.0
I.C.	16/18	88	9.8	16/26	62	14.9	8/21	38	17.1
I.P. + I.C.	13/14	93	11.1	14/21	67	15.9	11/23	48	16.7

* Mortality ratio: the numerator represents the number of mice that died, the denominator the number of mice inoculated.

† A.S.T.: average survival time, computed on the basis of a 21-day observation period, according to the method devised by Kerr and described by Bugher.[9]

and Reeves.[7] The tests were set up according to the technic previously described.[8] Undiluted sera were mixed with dilutions of virus and inoculated intraperitoneally in 0.03 ml amounts into mice 14 days of age; a group of 4 mice was used for each serum-virus mixture. The immune serum against the Hubbard strain of St. Louis virus neutralized 300,000 LD_{50} of the Winkler virus. The homologous Winkler immune serum neutralized >300,000 LD_{50} and the remaining sera neutralized 10 LD_{50} or less of the virus.

Neutralization tests were then done in the reverse direction, using the Hubbard strain of St. Louis virus and Winkler and Hubbard strain immune sera. Both sera neutralized >100,000 LD_{50} of the known St. Louis virus.

The Winkler virus was therefore regarded as a strain of St. Louis encephalitis virus. Inasmuch as the patient became ill within 6 to 7 days after his arrival in San Joaquin County, it is not impossible that he may have acquired his infection in Kern County. This is a point of some interest since, as mentioned above, Hammon and Reeves[1] in 1944 had found mosquitoes in Kern County to be infected with St. Louis encephalitis virus.

The Winkler virus was subsequently isolated in 2 additional instances in which the pathogenicity of the original human material was tested in mice of different ages. Mice of 14 days of age, and of 21 to 28 days and 6 to 7 weeks of age were inoculated intracerebrally (0.03 ml), intraperitoneally (0.1 ml), or by both routes, with a 10 percent suspension of human brain tissue; the animals were examined daily during a 4-week observation period to record the number of animals sick or dead.

The results of both experiments are summarized in Table I. As is indicated by the mortality data, the 14-day-old mice were much more highly susceptible to infection by the intraperitoneal route than were the older mice. Similarly, the 14-day-old animals were more susceptible to infection by the intracerebral or combined intracerebral-intraperitoneal routes than were the older animals; the difference in the mortality between the 2-week-old and the 6- to 7-week-old animals is especially striking, and quite different from that observed with a highly passaged laboratory strain of St. Louis virus inoculated intracerebrally.[10] The average survival times[9] also point to the decreasing susceptibility with age to infection by the several routes used. It appears, therefore, that for the isolation of certain viruses, at least, the use of young animals is desirable, and that if older animals are employed somewhat larger numbers should be inoculated to compensate for the lower degree of susceptibility. It would seem, also, that combined inoculation by 2 routes is a desirable procedure to enhance

[7] Hammon, W. McD., personal communication. The antiserum to this virus was kindly supplied by Dr. Hammon.

[8] Lennette, E. H., and Koprowski, H., *J. Immunol.*, 1944, **49**, 375.

[9] Bugher, J. C., *Am. J. Trop. Med.*, 1940, **20**, 809.

[10] Lennette, E. H., and Koprowski, H., *J. Immunol.*, 1944, **49**, 175.

TABLE II.
Influence of Age on Susceptibility of Hamsters to Infection with the Winkler Strain.

Inoculum*	Mortality ratios Age of test hamsters 2-3 days	4 days	6-7 days	10-13 days	15-16 days	6-8 weeks†	6+ mo‡
10% human brain	0/5	0/5	0/4			0/3	
10% 3rd passage mouse brain	9/9		10/10	13/13	16/29	1/12	1/10

* Inoculation was done by the intracerebral route; animals up to 16 days of age were given 0.03 ml, older animals 0.1 ml.
† Average weight of animals in this group was 53.8 g.
‡ " " " " " " " " 154 g.

the possibility of infection.

As mentioned above, hamsters inoculated with a suspension of the human brain survived without obvious signs of infection. Since hamsters are highly susceptible[5,6] to infection with laboratory passage strains of the St. Louis virus, the apparent lack of pathogenicity of the Winkler strain for this species was investigated. Hamsters of various ages were inoculated intracerebrally with 10 percent suspensions of the original human brain material or of 3rd mouse passage brains; animals up to 16 days of age received 0.03 ml, the older animals 0.1 ml. The results are brought together in Table II.

As is shown in Table II, hamsters as young as 2 to 3 days of age resisted infection with the original human brain material. After 3 passages in mice, however, the virus proved to be highly lethal for young hamsters but not for the older animals. Table II shows that 3rd mouse passage virus was uniformly lethal for hamsters up to 10 to 13 days of age and that the break in susceptibility occurred at about 15 days of age, since only 16 of 29 animals of 15 to 16 days of age succumbed to the infection. Animals 6 to 8 weeks of age or older were practically insusceptible; only 2 of 22 animals in this group died, one with and one without obvious evidence of illness on the day preceding death.

On the possibility that the local hamster strain might represent one resistant to the St. Louis virus, the Hubbard strain of virus, used in previous studies[6] on the susceptibility of hamsters to St. Louis virus, was titrated in animals 6 to 8 weeks of age. Serial tenfold dilutions of a 10 percent suspension of 100th mouse passage virus were prepared in serum broth and inoculated intracerebrally in 0.1 ml amounts; 2 animals were used for each dilution. The Hubbard strain proved to be uniformly lethal for local hamsters through a dilution of 10^{-7}, the highest tested. The resistance of hamsters older than 15 days to lethal infection with the Winkler virus was therefore considered to be due to the low pathogenicity, presumably associated with the low number of passages, of this strain, and not to a refractory state peculiar to the strain of hamsters used.

Hammon[11] has observed that freshly isolated strains of Western equine encephalomyelitis virus may show a complete lack of pathogenicity for guinea pigs, similar to that described here for the Winkler St. Louis virus in hamsters. Much of the recorded information on the susceptibility of various animal species to a given virus is based on the use of highly passaged laboratory strains. Failure of original source material to induce apparent infection in an animal species used for primary isolation of a virus, and generally regarded as susceptible to that virus, or of freshly-isolated, low-passage virus to produce overt infections in such species, should therefore be accepted with reservations and not used as an infallible criterion for the elimination or inclusion of certain viruses attendant on attempts at identification.

Summary. The virus of St. Louis encephalitis was isolated from a fatal human case of encephalitis in California. This represents the first isolation of this virus from any vertebrate host outside the endemic St. Louis area, and the second isolation of this virus in California.

[11] Hammon, W. McD., personal communication.

It is considered probable that the patient acquired his infection in Kern County, where Hammon and Reeves have shown the existence of naturally infected mosquitoes.

The pathogenic properties of the virus are described and discussed.

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Elimination in Human Feces of Infectious Hepatitis Virus Parenterally Introduced.*

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Experiments in the transmission of *infectious hepatitis* and *homologous serum jaundice* to human volunteers have revealed certain similarities and differences in the properties and distribution of virus, and route of infection in these 2 conditions. These points have been summarized recently by Neefe *et al.*[1] The etiologic agents of both conditions are filtrable, resistant to a temperature of 56°C for 30 minutes, and transmissible to human volunteers in serial passage.[2-5]

In contrast to these similarities are certain differences: namely; (1) the etiologic agent of infectious hepatitis is present in the serum and feces of patients in the acute phase of the naturally occurring or experimentally produced (by feeding) disease.[6-11] On the other hand attempts to demonstrate the etiologic agent of homologous serum jaundice in the *stools* of patients with this disease have been unsuccessful.[1,11,12] (2) Infectious hepatitis may be produced in human volunteers by *feeding* or parenteral inoculation of infectious material.[2,6-11,13] In contrast, while homologous serum jaundice has been produced experimentally by parenteral inoculation of infectious serum,[3-5,9,14] attempts to transmit this condition by feeding similar material have, with one exception, been unsuccessful.[1,5,12] This one exception is an experiment of MacCallum and Bauer[4] who produced the disease in a human volunteer by feeding infectious serum.

In view of these apparent differences it appeared possible that the *route of inoculation* might affect the distribution of virus and thereby constitute an artificial difference between these 2 conditions in experimental subjects. Failure to recover virus from the stool of patients with homologous serum jaun-

* Representing work done for the Commission on Neurotropic Virus Diseases, Army Epidemiological Board, Preventive Medicine Service, Office of the Surgeon General, United States Army.

Acknowledgment is made of the assistance and cooperation of the following agencies: Selective Service, Camp Operations Division and the Civilian Public Service Unit No. 140.

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12 Unpublished experiments of the author.

13 Neefe, J. R., and Stokes, J., Jr., *J. A. M. A.*, 1945, **128**, 1063.

14 Neefe, J. R., Stokes, J., Jr., Rheinhold, J. G., and Lukens, F. D. W., *J. Clin. Invest.*, 1944, **23**, 836.

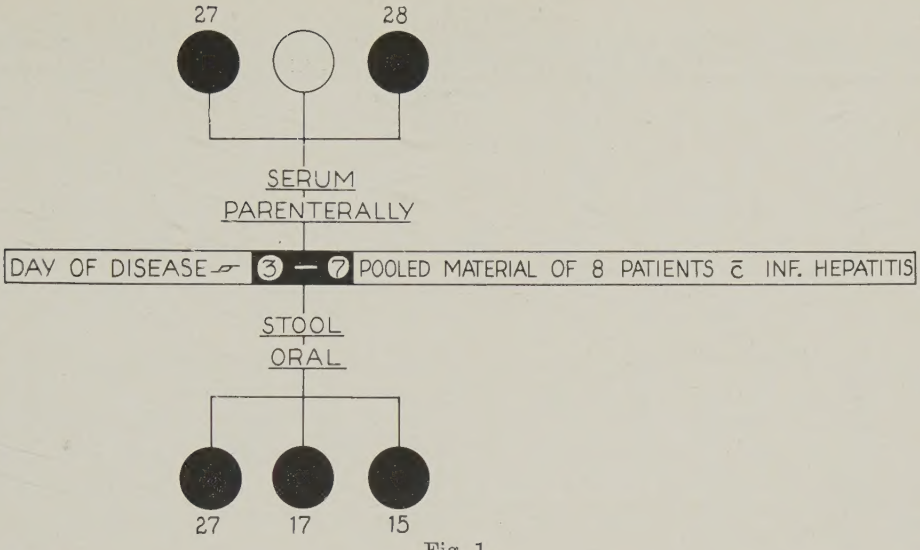

Fig. 1.

Illustration of results of administration to human volunteers of pools of serum and stool obtained during the acute phase (3-7th day) of 8 patients with infectious hepatitis experimentally induced by parenteral inoculation of infectious material. Open circle indicates a volunteer who was inoculated and failed to contract the disease; black circles indicate volunteers who contracted infectious hepatitis. The figure adjacent to the black circles represents the length of incubation period in days.

dice suggested that more information on the relation between this condition and infectious hepatitis might be gained if a similar situation were found when infectious hepatitis was produced by *parenteral inoculation.*

It is the object of this paper to determine whether a strain of infectious hepatitis virus, introduced *parenterally* into human volunteers, is eliminated in the feces of these same subjects when they contract the disease.

Materials and Methods. Virus. The strain of infectious hepatitis virus used in this laboratory was originally obtained from the stool of a U. S. Army soldier (BE) who contracted *epidemic infectious hepatitis* in Sicily in September 1943.[9] It has been through 4 passages in human volunteers to date. This agent is filtrable through an L2 Chamberland filter and withstands heating to 56°C for at least 30 minutes.[2] It has produced the disease in 27 out of 40 human volunteers (including this experiment) inoculated parenterally or orally with incubation periods ranging from 15 to 34 days.

Infectious materials employed in this experiment were pooled specimens of serum and of stools obtained during the acute (3-7th day) stage of disease experimentally induced in 8 volunteers by *parenteral inoculation* of this strain of infectious hepatitis. Stool and serum were always collected from the same patient on the same day. All material was stored at dry-ice box temperature for periods ranging from 3-7 months. Before use equal amounts of the same material from each volunteer were pooled and treated in the following manner: (a) serum was heated to 56°C for 30 minutes in a water bath; (b) stools were ground with sterile alundum and suspended in enough sterile 10/M buffered sodium phosphate to make a 10 percent suspension. This was centrifuged at 1500 r.p.m. for 30 minutes at room temperature to remove coarse particles. The supernate was removed and centrifuged at 6500 r.p.m. for 30 minutes at room temperature. This fairly clear supernate was removed and filtered through a Seitz EK filter at a pH of 7.0. The filtrate was then heated to 56°C for 30 minutes in a water bath. The serum and filtrates of stool were heated to eliminate the possibility of the presence of pathogenic bacteria. Both materials were sterile before administration.

TABLE I.
Duration and Height of Fever and Jaundice and Severity of Illness in 5 Human Volunteers with Experimentally Induced Infectious Hepatitis.

Volunteer	Inoculum	Source*	Amt cc	Route	Duration days			Maximum		Severity
					Incub. per.	Fever	Jaundice	Fever†	Serum Bilirubin mg. %	
BS	S	3-7th day disease (8 patients)	0.5	P	27	13	13	103.8	7.6	++
TR	S	"	0.5	P	28	5	14	101.0	2.5	+
SR	F	"	10	O	27	12	30	104.0	7.0	+++
HD	F	"	10	O	17	5	22	100.5	7.3	++
FW	F	"	10	O	15	10	14	102.5	7.4	++

S = Serum; F = Feces; P = Parenteral; O = Oral.
* Cf. Fig. 1. † Rectal temperatures are recorded.
Feces were administered as Seitz filtrates of 10% suspensions.

Three volunteers were fed the stool filtrate and 3 were inoculated sub- and intracutaneously with serum. The results are indicated in Fig. 1. It is apparent that virus is present in both the stool and serum of patients in the acute-phase of *parenterally induced* infectious hepatitis. Certain clinical data on the 5 volunteers who contracted the disease in this experiment are recorded in Table I.

Summary. 1. A strain of infectious hepatitis virus inoculated *parenterally* into 8 human volunteers was recovered from pools of serum and feces obtained from these same subjects during the acute-phase of their illness. These materials produced infectious hepatitis in 5 out of 6 healthy, human volunteers on reinoculation.

2. This recovery of virus from the stool of patients with infectious hepatitis induced by *parenteral inoculation* constitutes an apparent difference between this condition and homologous serum jaundice in which the etiologic agent has not been recovered from the stool up to the present time.

15277 P

Transplantability of Induced Granulosa Cell Tumors and of Luteoma in Mice. Secondary Effects of These Growths.*

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Granulosa cell tumors can be readily induced in the ovaries of mice by exposing the latter to X-rays.[1-3] A single dose of 87r given over the entire body to 44 mice approximately 6 weeks of age produced tumors in 31 mice. The induced ovarian growths

* These investigations have been supported by The Anna Fuller Fund, The International Cancer Research Foundation and The Jane Coffin Childs Memorial Fund for Medical Research.

1 Furth, J., and Furth, O. B., *Am. J. Cancer*, 1936, **28**, 54.

2 Furth, J., and Butterworth, J. S., *Am. J. Cancer*, 1936, **28**, 66.

3 Geist, S. H., Gaines, J. H., and Pollack, A. D., *Am. J. Obs. and Gyn.*, 1939, **38**, 786.

TABLE I.

	Males		Females		Total	
	Implanted	+	Implanted	+	Implanted	+
F1 mice						
Normal	23	11	21	8	44	19
X-rayed	5	1	20	12	25	13
Gonadectomized	10	8	15	12	25	20
Total	38	20	56	32	94	52
Parental (AK or Rf mice)	25	0	11	0	36	0

were of 3 main histological types: (a) tubular adenomas, (b) granulosa cell tumors, and (c) luteomas. The microscopic characteristics of these tumors have already been described and illustrated.[2]

Of 21 attempts at transfer of induced ovarian tumors, 13 proved successful. Induced tubular adenomas of 3 mice were successfully transplanted but only one has been carried in serial passages. This is a very slow growing tumor, becoming palpable (about 2 mm in size) after approximately 6 months (Strain VI). Two induced luteomas proved readily transplantable (Strains IX and XI). Cells of luteomata proliferate by mitotic division retaining the morphological characteristics of lutein cells in mice of both sexes. Mice bearing luteoma of Strain IX do not show evidence of hyperestrinization; the adrenal cortices undergo profound atrophy. These mice gained much weight, mainly by excessive deposition of fat in the normal fat depots.

The other transplantable ovarian tumors are granulosa cell growths. Strain I recently described[4] produces a cavernous dilatation of the liver sinusoids with only occasional secondary parenchymal damage which led to the suggestion that abnormal steroids may have been produced by the tumor and metabolized in the liver.[4]

Granulosa cell carcinoma, Strain III, now to be described, originated in an F1† mouse that had been given 175r at an age of 6 weeks and was subsequently painted with 0.5 percent methylcholanthrene dissolved in benzene (cf.[5]). Nine months after the irradiation a tumor felt at the site of the left ovary was removed and fragments of it were implanted into the subcutaneous tissue of this and 10 other F1 mice. When the donor animal died 7 weeks later the transplant measured 2x2x1 cm and the lung contained metastatic ovarian carcinoma.

The results of 9 subsequent transplantation experiments (4 successive passages) are summarized in Table I.

These figures indicate that the range of transmissibility of these ovarian tumor cells resembles closely that of normal cells (cf.[5]). At first the tumors grew slowly, becoming palpable only after about 4 months; but later their growth vigor increased and they became palpable within 6 to 8 weeks after transplantation. Metastases occurred in the liver and lungs.

The histological character of the tumor remained unaltered throughout the course of these passages. The tumors are composed of small granulosa-like cells which do not form follicles. They are widely separated by some pale staining material, perhaps their secretion. Necrosis with secondary calcification is common in the tumors and in such areas fibroblast-like cells can be seen which contain alkaline phosphatase granules in abundance and occasionally there is ossification.

There is continued estrus in most spayed or normal female mice bearing large tumors of Strain III, while in male mice the testes and seminal vesicles undergo profound atrophy. The thymus is markedly atrophic

4 Furth, J., and Boon, M. C., PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 112.

† Rf × AK hybrid.

5 Furth, J., and Boon, M. C., *Science*, 1943, **98**, 138.

in all tumor-bearing mice. In a few mice osteosclerosis of the femor, similar to that produced by estrogenic hormones[7] is noted. In addition, most, if not all, mice with large transmitted tumors had a cavernous dilatation of the sinusoids of the liver, spleen and adrenals. The weight of the liver was approximately 3 times normal in many mice, accounted for almost exclusively by an increment of blood. Although blood volume determinations have thus far not been made, the observations made suggest a marked rise in blood volume in mice exhibiting the liver change described. The cavernous dilatation of vessels was localized to viscera of the abdomen named. The vessels leading to the grafted tumor were also tremendously distended.

The mouse in which Strain III originated received both X-rays and methylcholanthrene. Extensive experience has shown that the latter alone does not produce ovarian tumors while X-rays alone have done so in almost as high a percentage of the mice as the combined treatment with X-rays and methylcholanthrene. However, a preliminary tabulation of our data shows that in mice receiving the combined treatment the tumors were larger, appeared sooner, were more readily transmissible and more apt to metastasize than those receiving X-rays only. In the latter, most tumors were of the tubular adenoma type, presumably derived from downgrowth of the germinal epithelium, while in the former, most tumors were of the granulosa or lutein cell types.

Summary. Eleven ovarian tumors, induced by X-rays have been transmitted in successive passages. Eight were of the granulosa cell types, 2 were luteomas and one was a tubular adenoma. Histological changes indicate that cells of Strain III secrete estrogens. Mice bearing tumors of this strain also have a cavernous dilatation of the sinusoids of liver, spleen and adrenals. In mice bearing large luteoma of Strain IX there is profound atrophy of the adrenal cortex.

6 Kaliss, N., and Robertson, T., *Genetics*, 1943, **28**, 78.

7 Gardner, W. U., and Pfeiffer, C. A., PROC. SOC. EXP. BIOL. AND MED., 1938, **37**, 678.

15278

A Method for Detection of Streptothricin in the Presence of Streptomycin.

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Streptomycin[1] and streptothricin,[2] two antibiotic agents having almost identical bacterial spectra,[3,4] are both produced by members of the actinomycetes, namely, *Actinomyces griseus* and *Actinomyces lavendulae* respectively. Because of this close relationship and of the still existing difficulty in the identification of the various members of the actinomycetes,[5] it is possible that strains of the organisms producing these 2 antibiotics might be confused. Such a confusion would be particularly undesirable when it is recognized that streptothricin is many times more toxic than streptomycin and in addition, has a distinct delayed toxicity.[6,7] Streptomycin,

1 Schatz, A., Bugie, E., and Waksman, S. A., PROC. SOC. EXP. BIOL. AND MED., 1944, **55**, 66.

2 Waksman, S. A., and Woodruff, H. B., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 207.

3 Robinson, H. J., Smith, D. G., and Graessle, O. E., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 226.

4 Robinson, H. J., Graessle, O. E., and Smith, D. G., *Science*, 1944, **99**, 540.

5 Waksman, S. A., and Henrici, A. T., *J. Bact.*, 1943, **46**, 337.

6 Rake, G., Hamre, D., Kavanagh, F., Koerber, W. L., and Donovich, R., *Am. J. Med. Sc.*, 1945, **210**, 61.

7 Robinson, H. J., Graessle, O. E., Gundel, M., and Silber, R. H., in press.

on the other hand, because of its relative non-toxicity and its active inhibition of gram-negative bacteria both *in vitro* and *in vivo*[3,8] has aroused a considerable amount of interest in the medical field. Realizing the difficulties which would be encountered if samples of streptomycin were to become contaminated with the more toxic streptothricin, it was considered essential that there be developed a means of detecting small amounts of streptothricin in the presence of large quantities of streptomycin. A method for differentiating between streptomycin and streptothricin has been announced recently.[9]

However, a more simplified microbiological test has been devised which can detect as little as 0.1% of streptothricin present as a contaminant in streptomycin. The procedure is based upon a modification of the Stebbins and Robinson[10] cup-assay for streptomycin. The organism used in the test is a strain of an unidentified gram-negative rod* which was found to be very resistant to streptomycin, but sensitive to streptothricin when tested for sensitivity by means of the agar-streak method. By this technic its growth was found to be uninhibited by 16,000 units† of streptomycin per ml, but to be inhibited by 7.5 units of streptothricin per ml. This strain is maintained by daily transfer in broth and a 6-hour culture, which has been incubated at 37°C, is used in the assay in the following manner: pour-plates are made using 10 ml of modified F.D.A. agar[10] which has been seeded while still fluid so as to contain finally a 10^{-4} dilution of the 6-hour culture. After the agar solidifies, penicylinders are set upon it, and the plates are then ready for use according to the regular cup-assay.

For the establishment of a standard curve from which to estimate the amounts of streptothricin present in the samples of streptomycin, a series of solutions are prepared as follows: (1) A stock solution of streptomycin is made in distilled water to contain 2000 units of streptomycin per ml of water. (2) A stock solution of streptothricin is prepared in distilled water to contain 40 units of streptothricin per ml of water. (3) Using solution No. 2, additional solutions are made to contain 20, 16, 8, 4 and 2 units of streptothricin per ml. (4) 0.5 ml quantities of the stock-solution of streptomycin (2000 units per ml) are then combined with 0.5 ml amounts respectively of the 6 solutions of streptothricin (40, 20, 16, 8, 4 and 2 units per ml) to give 6 standard solutions containing 1000 units of streptomycin per ml mixed with streptothricin to the extent of 20, 10, 8, 4, 2 and 1 units per ml. (5) A seventh standard solution consists of 1000 units of streptomycin per ml of water with no added streptothricin. (6) The test samples are made to correspond to the seventh standard solution; namely, to contain 1000 units of streptomycin per ml of distilled water. When prepared, the solutions of the test samples together with those of the 7 standards, which should always be run at least in duplicate with every new test, are put in the cups for assay against the streptomycin resistant strain. The plates are then incubated at 30°C for 16-18 hours, after which they are read for inhibition of growth as shown by the millimeters of zonation produced around the cups. Depending upon the degree of zonation produced, the amount of streptothricin present in the streptomycin can be detected. Table I shows the millimeters of zonation produced by the standard solutions. No sample of streptomycin, whether it was of low potency or of almost pure material, gave any zonation when used in a concentration of 1000 units

8 Jones, D., Metzger, H. J., Schatz, A., and Waksman, S. A., *Science*, 1944, **100**, 103.

9 Denkewalter, R. G., Cook, M. A., and Tishler, M., *Science*, 1945, **102**, 12.

10 Stebbins, R. B., and Robinson, H. J., PROC. SOC. EXP. BIOL. AND MED., 1945, **59**, 255.

* This organism was isolated by Dr. Bodenheimer from the trachea of a patient at the College of Physicians and Surgeons, Columbia University, New York City.

† One unit is that quantity of streptomycin which will just inhibit a given strain of *E. coli* (Waksman) in 1 ml of nutrient broth or agar. When measured in terms of antibacterial activity, one unit of streptothricin is equivalent to one unit of streptomycin.

TABLE I.
Diameters of Cleared Areas Produced by a Standard of Mixtures of Streptothricin and Streptomycin with Water as the Diluent.

Concentration of antibiotic agents	Zonation
1000 units streptomycin per ml only	0 mm
" " " " " plus 20 units streptothricin per ml	18.0 mm
" " " " " " 10 " " " "	16.5 "
" " " " " " 8 " " " "	15.5 "
" " " " " " 4 " " " "	13.5 "
" " " " " " 2 " " " "	12.0 "
" " " " " " 1 " " " "	8.5 "

of streptomycin per ml. However, concentrations of over 1000 units per ml produced moderate zones, for example 7.8 mm for 5000 units and 10.5 mm for 10,000 units per ml.

This culture has been maintained by daily transfer in this laboratory for a period of 8 weeks without showing any indications of changing in its response to streptomycin and streptothricin.

With this test one unit of streptothricin can be measured in the presence of 1000 units of streptomycin. Therefore it would be possible to detect as little as 0.1% of streptothricin if it were present as a contaminant in lots of streptomycin.

15279

Biological Conversion of n-Butyl Penicillin into a Chemotherapeutically Active Substance.

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Due to its rapid rate of absorption and excretion, it is impossible to maintain satisfactory blood levels of penicillin for prolonged periods after parenteral injection of an aqueous solution of penicillin. These characteristics necessitate either a frequent intermittent or a continuous type of injection. Attempts have been made to prolong the blood levels after parenteral injection of salts of penicillin by decreasing either their rate of absorption[1-4] or their rate of excretion.[5,6]

Since the rapid rate of elimination of penicillin following its parenteral injection is due in part to its extreme solubility in aqueous phases, it would appear advantageous to modify the penicillin molecule in such a manner as to form water insoluble derivatives. This goal has been accomplished by the production of various esters of penicillin. Meyer and his co-workers have reported the preparation of a number of such esters including the methyl, ethyl, n-butyl and benzhydryl esters.[7] All of these compounds were de-

* On leave of absence from the May Institute for Medical Research and the Department of Bacteriology of the College of Medicine, University of Cincinnati, Cincinnati, Ohio.

1 Fisk, R. T., Foord, A. C., and Alles, G., *Science*, 1945, **101**, 124.

2 Parkins, W. M., Wiley, M., Chandy, J., and Zintel, H. A., *Science*, 1945, **101**, 203.

3 Romansky, M. J., and Rittman, G. E., *Bull. U. S. Army Med. Dept.*, 1944, **81**, 43.

4 Trumper, M., and Hutter, A. M., *Science*, 1944, **100**, 432.

5 Beyer, K. R., Peters, L., Woodward, R., and Verway, W. F., *J. Pharmacol.*, 1944, **82**, 310.

6 Rammelkamp, C. H., and Bradley, S. E., PROC. SOC. EXP. BIOL. AND MED., 1943, **53**, 30.

7 Meyer, K., Hobby, G. L., and Dawson, M. H., PROC. SOC. EXP. BIOL. AND MED., 1943, **53**, 100.

TABLE I.
The Protective Action of Subcutaneous Injections of n-Butyl Penicillin Against Streptococcal Infections in the Mouse.

Wt of Ester injected, mg	Dilution of culture inj.	No. of mice inj.	No. of mice survived	No. of mice died
0	10^{-6}	3	1	2
	10^{-5}	3	0	3
	10^{-4}	3	0	3
0.1	10^{-6}	3	2	1
	10^{-5}	3	2	1
	10^{-4}	3	1	2
0.5	10^{-6}	3	3	0
	10^{-5}	3	3	0
	10^{-4}	3	3	0

Note. Doses of 1.0 mg and 5.0 also gave complete protection.

scribed as insoluble in neutral or slightly alkaline buffers but were quite soluble in fat solvents. They were almost devoid of any activity *in vitro* but apparently could be hydrolyzed in alkaline solution to regenerate an active principle, presumably the non-esterified penicillin.[8]

In contrast to their relative inactivity *in vitro*, the ethyl and n-butyl esters were demonstrated to be effective chemotherapeutic agents in mice infected with large doses of virulent hemolytic streptococci.[7] Thus, single doses of these esters, given either orally or subcutaneously, protected mice against intraperitoneal injections of streptococci. The protective action of methyl penicillin in mice infected with spirochetes has also been demonstrated.[9] It appeared, therefore, that these esters were hydrolyzed *in vivo* with the slow liberation of active penicillin. Such results suggested the possibility of the successful use of penicillin esters as chemotherapeutic agents in man.

More recently, following the preparation of the benzyl ester of penicillin,[10] reference was made to unpublished results[11] of the successful use of this ester in clinical infections and high promise was held forth for the effectiveness of penicillin esters against infections in man. In contrast to these results, previous work in our own laboratory had indicated that higher mammals were unable to effect the conversion of alkyl penicillin esters into chemotherapeutically active substances. Accordingly, we report a few preliminary experiments obtained with the n-butyl ester in the dog, monkey and man.

Methods and Results. Four of the butyl penicillin samples were dark brown viscid oils; one was a yellowish powder. Their *in vitro* activity was tested by dissolving a weighed amount in absolute alcohol and diluting further with broth. Their activity was then assayed against the C203 MV strain of hemolytic streptococcus and all samples showed an activity of 5 to 10 units per mg as opposed to 600 to 1100 units per mg of the original free penicillin from which they had been prepared.

In work with dogs and monkeys, weighed amounts of the ester were dissolved in absolute alcohol and diluted with 4 parts of propylene glycol. The alcohol-glycol solutions were then injected without further sterilization. Preliminary to injection into man, the ester was dissolved in the alcohol-glycol mixture as described above and sterilized by filtration through a small sterile sintered glass filter. The filter was washed with small amounts of absolute alcohol until the washings showed no traces of color. The solution was concentrated in the cold under high vacuum until alcohol was no longer detectable. The residue was then assumed to contain the total amount of ester originally

8 Hickey, R. J., *Science*, 1945, **101**, 462.

9 Richardson, A. P., Walker, H. A., Loeb, P., and Miller, I., *J. Pharm.*, 1945, **85**, 23.

10 Cavallito, C. J., Kirchner, F. K., Miller, L. C., Bailey, J. H., Klimek, J. W., Warner, W. F., Sutler, C. M., and Tainter, M. T., *Science*, 1945, **102**, 150.

11 Gamble, T. O., Miller, L. C., and Tainter, M. L., *Am. J. Obst. and Gynec.*, in press.

TABLE II.
Recovery of Free Penicillin from the Urine and Blood after Subcutaneous Injection of n-Butyl Penicillin.

Animal	Wt of Ester inj. mg	Duration of urine collection after inj. of Ester, hr	Total free penicillin recovered in urine units	Serum penicillin levels hr after inj. 1	2	3	4	8	12	24
Dog	100	48	22	0	*	0	*	0	*	0
Dog	200	48	31	*	0	*	0	*	0	*
Monkey	100	36	4	*	*	*	*	*	*	*
Monkey	200	48	17	*	*	*	*	*	*	*
Man	200	48	14	0	0	0	0	0	0	0
Man	200	48	46	0	0	0	0	0	0	0

* Indicates no determination made.

weighed out and was used, in appropriate quantities, for parenteral injection into man.

Penicillin assays on blood samples and urine specimens were performed by a serial dilution technic. Since it was believed the urine assays offer a more reliable index of the total absorption of penicillin,[12] such assays were used to indicate the degree of conversion of the ester in the body.

We were able to confirm the results of Meyer, Hobby and Dawson[7] in regard to the chemotherapeutic activity of n-butyl penicillin in mice. For this purpose, mice weighing approximately 20 g were given intraperitoneal injections of 0.5 ml of a 10^{-4}, 10^{-5} and 10^{-6} dilution of a 15 hour culture of the virulent C203 MV strain of the Group A *Streptococcus*. One-half hour later, the treated animals each received a single subcutaneous injection of the propylene glycol solution of the ester. The results given in Table I indicate that a single injection of 0.5 mg of ester protected all of the mice against 0.5 ml of a 10^{-4} dilution of the culture. Only moderate protection was afforded by 0.1 mg of ester.

No studies on the chemotherapeutic activity of the butyl penicillin were performed on the dog or the monkey, but these animals were used to investigate the pharmacology of this compound and to determine their reaction to large doses subcutaneously.

Two dogs weighing approximately 5 and 7 kg were given subcutaneous injections of 100 mg and 200 mg, respectively, of the ester. The injections apparently produced a transient moderate local irritation. Observations of both dogs failed to reveal any evidences of a systemic reaction to the injections. Blood and urine assays for free penicillin were performed and the results in Table II indicate failure to produce demonstrable blood levels or more than minute quantities of excreted penicillin.

Similarly, 2 monkeys *(M. mulatta)* weighing approximately 4 kg received 100 and 200 mg each of the butyl penicillin subcutaneously. As was the case in the dogs, the monkeys failed to evidence any reactions to the injections other than a transient mild local irritation. Blood assays were not performed. However, as in the dogs, composite urine samples did not reveal appreciable excretion of free penicillin (Table II).

Inasmuch as the dogs and monkeys appeared to tolerate the injections of penicillin ester, it was considered safe to determine their effect in man. In order to investigate chemotherapeutic activity of the ester, male patients with acute gonorrheal urethritis were chosen since this disease is so sensitive to the activity of penicillin. Two such patients were each given subcutaneous injections of 100 mg of the ester followed 6 hours later by another similar injection. None of the injections produced immediately more than an extremely mild irritation. The following day, however, the site of the injections was swollen, hot and very tender. In addition, there was a moderate febrile reaction accompanied by some malaise. Both the systemic and local reactions had disappeared 48 hours later. Due to the impurity of the preparation, it was difficult to assess the

[12] Pedrick, R. F., and Broh-Kahn, R. H., *AAF School of Aviation Medicine Res. Rep.*, 1945, 388-1.

role of the solvent or of the impurities in this moderately toxic reaction. Urine and blood assays were performed and the effect on the disease determined. The results of the assays (Table II) again revealed little or no free penicillin. Clinically, the urethral discharges showed no improvement and smears and cultures continued to reveal the presence of viable gonococci. These observations were continued for 3 days after the injections. If the greater part of the ester had been hydrolyzed, the quantity of free penicillin so liberated might have been expected to produce a demonstrable improvement in the clinical condition. At the end of this period, both patients were treated with 500,000 units of oral penicillin[13] and their disease responded satisfactorily, thus affording evidence that the infecting strains were not or had not become refractory to penicillin. In order to determine whether the method of preparation of the solution of ester for injection into man had resulted in its inactivation, the same solution was later demonstrated to protect mice against the intraperitoneal injections of 0.5 ml of a 10^{-4} dilution of a 15 hour culture of a virulent hemolytic streptococcus.

Discussion. There would appear to be little doubt that subcutaneous injections of butyl penicillin protect mice against infections by hemolytic streptococci. This protection obviously cannot be attributed to any impurity of unchanged penicillin since 1 mg of the ester contains a maximum of 10 units of activity. Inasmuch as 0.5 mg is sufficient to protect mice, the protective dose contains a maximum of 5 units of free penicillin. Approximately 200 units of free penicillin are required for protection of the mouse against lethal doses of hemolytic streptococci.[14,15]

The possibility exists either that the esters *per se* exert a chemotherapeutic action or that they, after injection, are converted in the body into active substances. It appears doubtful that the esters themselves are the actual chemotherapeutic substances since they failed to display any activity against human gonococcal infections and since they are almost devoid of activity *in vitro*. Accordingly, it appears most plausible to assume that, subsequent to absorption, the esters are converted into the active agent. The most probable conversion would undoubtedly be a regeneration of free penicillin. *In vitro* studies[16] have already demonstrated that this reaction occurs. The most reasonable explanation for the mechanism of the activity of the ester in mice would be its constant and somewhat slow conversion, in the body, to free penicillin. If this conversion occurred at an appropriate rate, adequate levels of free penicillin might be maintained in the blood for prolonged periods following a single injection of ester. Once the hydrolysis had occurred the resulting penicillin would be expected to be excreted in the urine at its normal rate.

On the other hand, following subcutaneous injections of the ester in the dog, the monkey and in man, the observations of this study indicate the inability to recover appreciable quantities of penicillin in either the blood or the urine. It would seem, therefore, that the tissues of these mammals are unable to effect the conversion of the ester into free penicillin at an appreciable rate.

In general, the velocity of hydrolysis of aliphatic esters by tissues varies inversely with the molecular weight of the esterifying alcohol.[17] It was therefore believed that efforts should be directed towards investigation of simpler esters. However, Richardson *et al.*[18] report results with both the methyl and benzyl esters of penicillin comparable to our own findings with the n-butyl ester. In view of the fact that the benzyl radical behaves as an aliphatic derivative, it would have been somewhat surprising to learn that tissues unable to hydrolyze methyl and butyl penicillin could effect the hydrolysis of the

[13] Broh-Kahn, R. H., *AAF School of Aviation Medicine Res. Rep.*, 1945, 404-1.

[14] Hobby, G. L., Meyer, K., and Chaffee, E., PROC. SOC. EXP. BIOL. AND MED., 1942, **50**, 285.

[15] Robinson, H. J., *J. Pharm.*, 1943, **77**, 70.

[16] Broh-Kahn, R. H., and Smith, P. K., unpublished observations.

[17] Hass, G. M., *Arch. Path.*, 1938, **26**, 1183.

[18] Richardson, A. P., Walker, H. A., Miller, J., and Hansen, R., PROC. SOC. EXP. BIOL. AND MED., 1945, **60**, 272.

higher benzyl homologue.

Summary and Conclusions. 1. A subcutaneous injection of butyl penicillin conferred marked protection against hemolytic streptococcal infections in the mouse. 2. Subcutaneous injections of 200 mg exerted no appreciable effect on gonococcal infections in 2 men. 3. Following subcutaneous injections of butyl penicillin into the dog, the monkey, and man, appreciable quantities of penicillin were not recovered in the blood or urine. 4. The subcutaneous injection of from 100 to 200 mg of butyl penicillin produced no evidences of marked toxicity in the dog or the monkey. Two men reacted with evidences of moderate toxicity. 5. The tissues of the mouse can convert the chemotherapeutically inactive butyl penicillin into a chemotherapeutically active substance, presumably penicillin. The tissues of man, the monkey, and the dog cannot effect this conversion at an appreciable rate.

Dr. Karl Meyer kindly furnished us with the butyl penicillin used in this study and supplied the data concerning the *in vitro* activity against the streptococcus.

15280

Influence of Iron Salts on the Toxicity of Lead.

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The relationship between the toxicity of lead and the dietary level of other elements has been studied for many years. The extensive literature has been reviewed by Cantarow and Trumper.[1] Calcium and phosphate have been especially implicated in modifying the effects of oral administration of lead salts. The influence of iron compounds on the development of lead poisoning has received little study. Our attention was directed to this problem during the course of work on blood formation in various toxic states. In the present study it was found that iron salts prevented the weight depression and anemia in rats fed diets containing lead acetate.

Experimental. Albino rats of Wistar and Osborne and Mendel strains were fed the experimental diets at weaning. Feeding was *ad libitum* except in Exp. 2. The animals were weighed at intervals of 4 to 7 days. Tail blood was used for micro-determinations of the hematocrit (Van Allen) and for hemoglobin determinations by the oxyhemoglobin method of Sanford *et al.*[2] Blood smears were fixed in absolute methanol and stained for 1½ hours with dilute giemsa solution buffered to pH 7.2. The total number of basophilic red cells per 1000 red cells were counted with the aid of a Whipple disc.

Effects of ferric citrate and ferrous sulfate. In a preliminary study with semipurified diets it was found that supplements of ferric citrate and copper sulfate protected against the depression in weight gain and anemia of lead poisoning. Subsequent tests showed that ferric citrate alone was equally effective.

Table I shows the results of 2 experiments in which male weanling rats were used.*

[1] Cantarow, A., and Trumper, M., *Lead Poisoning*, The Williams and Wilkins Co., Baltimore, 1944.

[2] Sanford, A. H., Sheard, C., and Osterberg, A. E., *Am. J. Clin. Path.*, 1933, **3**, 405.

* The percentage composition of the basal diet for these and all remaining experiments was as follows: purified casein (Smaco) 30.0, hydrogenated cottonseed oil (Crisco) 10.0, U.S.P. cod liver oil 5.0, choline chloride 0.2, salt mixture 1.9, cane sugar 52.9. In addition, the vitamin supplement (in mg per kilo) was thiamine hydrochloride 10, nicotinic acid 40, pyridoxine hydrochloride 10, calcium pantothenate 40, and riboflavin 20. Each rat received a weekly oral supplement of 3 mg of

TABLE I.
Effect of Ferric Citrate on the Toxicity of Lead.

	Added to 100 g diet				
	Lead* g	Ferric-citrate g	No. of rats	Mean wt† g	Mean hemoglobin‡ g/100 cc
Exp. 1	—	—	12	159 (117-196)	15.1
	0.03*	—	8	106 (85-144)	9.9
	0.03*	1.13	8	148 (111-170)	16.0
Exp. 2	—	—	10	149 (123-187)	16.6
	0.03*	—	10	126 (85-156)	12.1
	0.03*	1.13	10	141 (115-180)	14.8
	0.03*	1.47	10	142 (112-172)	15.5

* After 31 days on test, the lead content of the diets was increased to 0.09%.
† After 27 days in Experiment 1 and 56 days in Experiment 2.
‡ Determinations done after 66 days in Experiment 1 and after 75 days in Experiment 2.

TABLE II.
Effect of Ferric Citrate and Ferrous Sulfate on the Toxicity of Lead.

Added to 100 g basal diet	Mean wt after 51 days g	Mean hemoglobin after 48 days g/100 cc
(1)	213.4	15.8
(2) 0.09 g Pb (as Lead Acetate)	81.5	10.1
(3) 0.09 g Pb + 1.13 g Fe Citrate	168.0	15.3
(4) 0.09 g Pb + 0.25 g Fe Citrate (0.84 mmols)	130.4	12.9
(5) 0.09 g Pb + 0.2 g $FeSO_4$ (1.32 mmols)	133.3	15.5

In Exp. 1, the differences between the mean weights for the rats on basal diet with lead and the other two groups were examined by Fischer's *t*-technic[4] and found to be significant with a P value below 0.01. With the pair-feeding technic (Exp. 2) differences in weight gains were small enough to be disregarded. This has been observed before among lead poisoned rats.[5] But a statistically significant anemia, preventable by generous additions of ferric citrate, was obtained.

In Exp. 3, (Table II), 50 male weanling rats were divided into 5 groups equal with respect to average weight and litter distribution. It was found that an addition of 0.25 g of ferric citrate was less effective than 1.13 g in opposing the toxicity of lead. Ferrous sulfate also had some protective action. The differences in average weight gains and hemoglobin concentrations between the group given basal diet containing lead and all of the other groups were analyzed statistically and found to be significant.

Ineffectiveness of sodium citrate. Experiments were carried out to determine whether

α-tocopherol in 0.03 cc ethyl laurate. The salt mixture (in g) was composed of: NaCl, 138; $CaCO_3$, 200; $MgCO_3$, 50; KH_2PO_4, 424; $MgSO_4 \cdot 7H_2O$, 65.6; KCl, 224; $MnSO_4 \cdot H_2O$, 0.784; NaF, 2.0; KI, 0.16; $Al_2(SO_4)_3 \cdot K_2SO_4 \cdot 24H_2O$, 0.622; $CuSO_4 \cdot 5H_2O$, 11.95; Ferric Citrate, 16.55. This salt mixture was designed to make a low calcium-high phosphorus ratio in the completed diet. According to the work of Grant *et al.*[3] such a diet favors the deposition of lead in bone, liver and kidney. Samples of the ferric citrate used were analyzed and found to contain 18.1% of iron.

[3] Grant, R. L., Calvery, H. O., Laug, E. P., and Morris, H. J., *J. Pharm. and Exp. Therap.*, 1938, **64**, 446.

[4] Fischer, R. A., *Statistical Methods for Research Workers*, Oliver and Boyd, London, 1936.

[5] Baernstein, H. D., and Grand, J. A., *J. Pharm. and Exp. Therap.*, 1942, **74**, 18.

TABLE III. Effect of Ferric Citrate and Sodium Citrate on Depression of Growth Rate, Anemia and Red Cell Polychromasia of Lead Poisoning.*

Group	Added to 100 g, basal diet: Lead g	Ferric citrate g	Sodium citrate g	Avg wt g, Days on diet: 0	25	51	65	Hemoglobin g/100 cc, Days on diet: 26	61	Hematocrit %, Days on diet: 26	61	Polychromatic red cells per 1000r.b.c. after 62 days avg no. and range
A	—	—	—	43.2	137.3	230.3	239.4	14.2	16.1	45	49	3.8 (2-6)
B	0.09	—	—	40.9	89.7	128.2	143.2	13.1	10.5	44	39	49.0 (12-151)
C	0.09	1.13	—	39.0	127.1	192.3	203.1	14.7	16.4	45	51	8.2 (0.1-23)
D	0.09	—	1.15	41.3	92.2	128.6	137.1	12.5	10.9	39	40	62.6 (16-180)

Significance of Difference Between Means†

	Groups compared (first term greater than second term): A-B	A-C	C-A	A-D	C-B	B-D	D-B	C-D
Weight gain, 51 days	$P<0.001$	$P<0.02$	———	$P<0.001$	$P<0.001$	$P>0.3$	———	$P<0.001$
Hemoglobin, 61 days	$P<0.001$	- - — -	$P>0.5$	$P<0.001$	$P<0.001$	———	$P>0.6$	$P<0.001$

* Each group contained 10 rats. One rat in group A died after 24 days on experiment and one rat in group B died after 57 days on experiment.

† These P values indicate that there was no statistical significance between the weight gains or hemoglobin values of the rats receiving lead alone and those of rats getting sodium citrate added to the lead salt. The iron treated rats had hemoglobin levels not significantly different from those on basal diet. All other differences between groups were significant. Weight gains for a period of 51 days were analyzed because this covered the phase of most rapid growth for the control rats.

the citrate radicle in ferric citrate had any protective effects. It was first ascertained that sodium citrate could be fed to weanling rats at a level of 1.15 g added to 100 g of diet without depressing the rate of growth. This was equivalent to 1.13 g of ferric citrate.

In a preliminary experiment it was found that sodium citrate did not protect against the growth depressant effect of lead. Young rats were fed the basal diet, the leaded basal diet or the leaded basal diet with 1.15 g of sodium citrate added to every 100 g of diet. In one test 5 rats were placed in each group and in a second test there were 6 rats in each group. In both tests it was found that after 30 days the mean weight of the sodium citrate treated rats was approximately 10 g less than that of the rats receiving only lead.

In the next experiment 40 male weanling rats of the Wistar strain were divided into 4 groups equal with respect to litter distribution. The diets and experimental results are shown in Table III.

Discussion. In these experiments iron salts interfered in some way with the toxicity of lead. This was deduced from the fact that the depression of growth rate, anemia and red cell polychromasia, were reduced or absent in rats given supplements of ferric citrate or ferrous sulfate.

The anemia of the leaded rats, although prevented by supplements of ferric citrate, was not related to an inadequate iron content of the basal diet. The rats on the unleaded basal diet did not become anemic even when pair fed against those receiving lead.

It may be of interest to compare the content of ferric citrate in our protective diets with that obtained by one of the better known salt mixtures as ordinarily used. The McCollum-Davis salt mixture 185,[6] when fed at a 5% level, contributes 0.16% of ferric citrate to the diet. In the present study a supplement of approximately 0.25% of ferric citrate, did not bring about normal hemoglobin concentrations in leaded animals, although it had some beneficial effect. A level of 1.13% prevented the anemia entirely.

It is of interest that Miyasaki[7] found that a colloidal solution of 2% dialyzed ferric oxide reduced the absorption of lead in mice although a suspension of ferric hydroxide was ineffective. The mechanism of action of iron salts in counteracting the toxicity of orally administered lead in these studies has not been established. However, experiments in progress indicate a much lower lead content of tissues of rats whose diet contained supplements of iron citrate. A possible explanation for these findings would be an interference by iron citrate with the absorption of lead. Further studies including the use of radioactive lead (radium D) for tracing the absorption of lead in the presence of iron compounds are planned. The possibility of an interference with the absorption of iron by lead also merits investigation.

Summary. Ferric citrate as 1.12% of the diet protected young rats against the weight loss, anemia and red cell polychromasia of lead poisoning. One-fourth of this amount was less effective. About 0.2% of ferrous sulfate had a protective action. Sodium citrate was ineffective.

[6] McCollum, E. V., and Davis, M., *J. Biol. Chem.*, 1918, **33**, 55.

[7] Miyasaki, S., *Arch. Exp. Path. u. Pharmakol.*, 1930, **150** (1/2), 39.

Miss Virginia T. Porterfield, Mrs. Evelyn G. Peake and Miss D. Louise Odor assisted with the experiments and helped to perform the statistical analyses.

15281

Influence of Certain Substances on Activity of Streptomycin. I. Modifications in Test Medium.

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It was previously shown[1] that the amount of streptothricin required to inhibit the growth of the test organism, *Klebsiella pneumoniae*, in 1% tryptone broth varied with the lot of tryptone employed in preparing the broth. To this extent we had found that streptomycin behaves in a like fashion. Wallace *et al.*[2] have reported that suspensions of *Eberthella typhosa* showed marked drops in viable cell counts when exposed to streptomycin in the presence of "nutrient broth" and "nutrient broth diluted with an equal amount of water," whereas in brain heart infusion broth the same concentration of streptomycin had no apparent effect. *Staphylococcus aureus* suspensions under similar conditions showed marked drops in viable cell counts in all 3 media during the first 6 hours of exposure to streptomycin, but grew out heavily in brain heart infusion broth during the interval between the 6th and 9th hours of exposure.

The present paper deals with the growth-inhibiting activity of streptomycin in (a) a culture medium containing only tryptone and water or tryptone, dextrose and water, and (b) an enriched medium containing sodium thioglycollate.

A. The Activity of Streptomycin in Culture Media Containing Varying Amounts of Tryptone and Dextrose. In the paper already mentioned[1] it was shown that when streptothricin was added to 2 ml of 6-hour broth cultures of *K. pneumoniae* diluted 10^{-6} in broth consisting of 1% tryptone (or tryptose) and water, from 0.08 to >0.16 units of streptothricin per ml of broth were required to cause complete inhibition of growth, as observed after 17 hours incubation at 37°C. The minimum inhibiting concentration of streptothricin varied with the lot of tryptone or tryptose used. As indicated above, the action of streptomycin on *K. pneumoniae* was similarly affected by the various lots of tryptone. In the present paper it will be shown that even when a single lot of tryptone is used for preparing test media, the minimum inhibiting concentration of streptomycin will vary considerably with the concentration of tryptone used. Further, if dextrose is also added to the medium, the minimum inhibiting concentration will again be increased.

Procedure. Media were prepared containing from 0.5 to 1.0% tryptone (from a given lot) to which were added from 0 to 3% dextrose. A 6-hour culture of *K. pneumoniae* was diluted 10^{-6} in each of these media. The diluted cultures were dispensed into tubes in 2 ml volumes to which were then added increasing amounts of a standard streptomycin solution containing 2.0 units/ml according to the assay procedure referred to above.[1] The minimum inhibiting concentration (MIC) of streptomycin in each medium determined in this fashion is shown in Table I and Fig. 1.

Thus it is seen that as the concentration of tryptone rises in the test medium, the minimum inhibiting concentration of streptomycin increases. To a lesser extent addition of dextrose to the medium also raises the MIC, and interestingly has a more marked effect when added to 1.0% tryptone than to 0.50% tryptone. The addition of increasing amounts of dextrose to tryptone broth causes decreasing end pH values in the broth on autoclaving. Waksman and Schatz[5] have shown that lowering pH of agar lowers the

[1] Donovick, R., Hamre, D., Kavanagh, F., and Rake, G., *J. Bact.*, 1945, **50**, 623.

[2] Wallace, G. I., Rhymer, I., Gibson, O., and Shattuck, M., Proc. Soc. Exp. Biol. and Med., 1945, **60**, 127.

[5] Waksman, S. A., and Schatz, A., *J. Am. Pharmaceut. Assoc.*, 1945, **34**, 273.

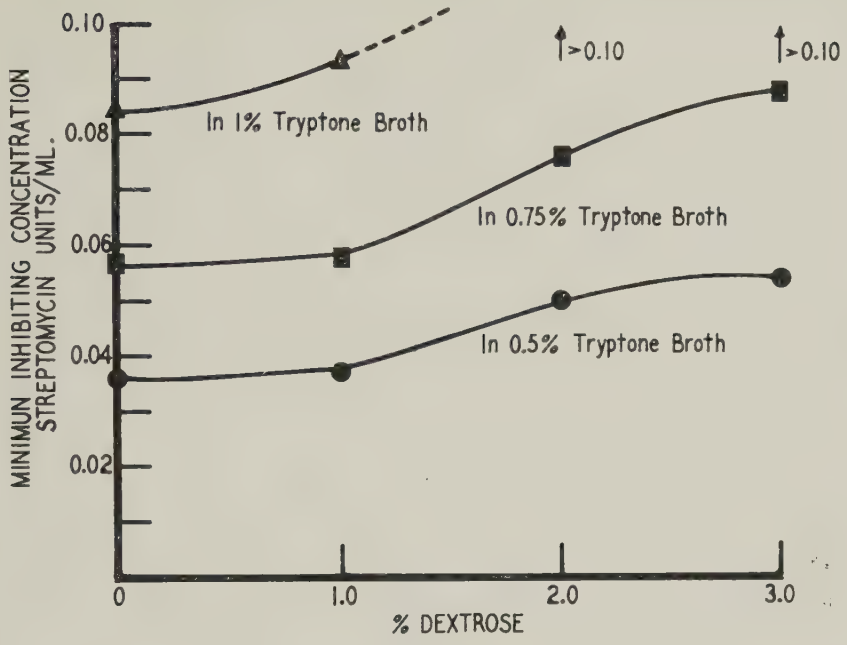

FIGURE I

Effect of Dextrose and Tryptone in Media on Minimun Inhibiting Concentration of Streptomycin for K. pneumoniae

activity of streptomycin in the cup-plate test. We have observed similar effects in broth. Thus the interference caused by dextrose may be due in part to the effect on pH. However this does not explain the finding that dextrose causes greater interference in broth containing 1.0% and 0.75% tryptone than in broth containing 0.5% tryptone since the pH drop with increasing dextrose was identical in all 3 concentrations of tryptone.

*B. The Activity of Streptomycin in an Enriched Medium Containing Sodium Thioglycollate.** Denkewalter, Cook and Tishler[3] have reported that streptomycin and streptothricin are "not inactivated to any significant extent by thioglycollic acid." Their data, however, shows that where a phosphate buffer control of streptomycin assayed at 49 units/ml, control solutions containing in addition 0.50, 2.50 and 3.0 mg respectively of thioglycollic acid per ml, assayed at 31, 39 and 26 units/ml respectively. Similar results were obtained with streptothricin.

In the present studies we have compared the activity of streptomycin in broth consisting of 0.75% tryptone with that in thioglycollate broth containing the following ingredients:†

Cystine	0.75 g
NaCl	2.5 "
Dextrose	5.0 "
Agar	0.75 "
Yeast Extract	5.0 "
Pancreatic digest of casein	15.0 "
Sodium thioglycollate	0.5 "
Resazurin (0.1%)	1 ml
Water to make 1000 ml	

* At the time that this paper was in proof Bondi, Dietz and Spaulding[4] published similar findings on the effects of sodium thioglycolate and other reducing substances on the action of streptomycin.

[3] Denkewalter, R., Cook, M. A., and Tishler, M., *Science*, 1945, **102**, 12.

[4] Bondi, A., Jr., Dietz, C. A., and Spaulding, E. H., *Science*, 1946, **103**, 399.

† Described in a circular from the National Institute of Health (January 15, 1945) revising the culture medium required for sterility testing of biologics.

TABLE I.
Effect of Tryptone and Dextrose on Minimum Inhibiting Concentration of Streptomycin.

% Tryptone	% Dextrose			
	0	1.0	2.0	3.0
		MIC* units/ml		
0.50	0.036	0.037	0.050	0.054
0.75	0.056	0.058	0.076	0.088
1.0	0.084	0.093	>0.10	>0.10

* Minimum inhibiting concentration of streptomycin.

TABLE II.
Comparison of Minimum Inhibiting Concentrations of Streptomycin in Tryptone and Thioglycollate Broths.

MIC in tryptone broth u/ml*	MIC in thioglycollate broth			
	Broth No. 1†		Broth No. 2‡	
	Age of broth§ days	MIC u/ml	Age of broth§ days	MIC u/ml
0.055	3	1.48	1	>2.98
0.053	6	1.37	2	2.41
0.051	7	1.25	3	2.81
0.057	15	1.04	7	1.52
0.059	16	0.99	8	1.35
0.051	23	1.06		
0.045	24	0.69		
0.055	42	0.63		
0.056	43	0.55		

* Units per ml.

† Prepared in this laboratory.

‡ Prepared from dehydrated broth obtained from Baltimore Biological Laboratory, Baltimore, Md.

§ Age of broth indicates the interval between the time of preparation of the broth and the time when the broth was used. During this interval the flasks of broth remained at room temperature.

The media were sterilized by autoclaving at 15 pounds pressure for 20 minutes. After sterilization the pH of both types of media was 7.0-7.2.

A single lot of tryptone was used throughout for preparing the tryptone broth. Two thioglycollate broths, containing the given ingredients, were employed. One was prepared in this laboratory while a second was obtained in a dehydrated form from an outside source. The minimum inhibiting concentrations of streptomycin per ml of test broth are shown in Table II. For the tests a 6-hour culture of *K. pneumoniae* diluted 10^{-6} in the given broth was used.

As is shown in Table II, approximately 27 times more streptomycin was required to inhibit the growth of *K. pneumoniae* in thioglycollate broth No. 1, 3 days after the broth was prepared, than in 0.75% tryptone broth. Forty days later the ratio of MIC values in these 2 broths had dropped to 10:1. In thioglycollate broth No. 2, when used one day after preparation, the minimum inhibiting concentration was 54 times greater than that in tryptone broth. This ratio decreased to about 24.5:1 after the thioglycollate broth had stood for 8 days at room temperature. It was further found that in a broth containing the same ingredients as thioglycollate broth No. 1 in Table II, except that sodium glycollate was substituted for sodium thioglycollate, the minimum inhibiting concentration of streptomycin was about 10 times greater than that in 0.75% tryptone broth. This compares to thioglycollate broth No. 1 after it had stood at room temperature for 43 days.

Thus it is seen that the incorporation of sodium thioglycollate in a culture medium greatly interferes with the activity of streptomycin. Since sodium thioglycollate is readily

oxidized, this interference with streptomycin activity gradually decreases as the broth stands. What may be called the baseline of interference of the broth, which is reached after some 40 days of standing in one thioglycollate medium we have used, is similar to the interference caused by the same medium containing sodium glycollate instead of sodium thioglycollate.

It is interesting that the action of streptothricin is affected to a similar or perhaps even greater extent in thioglycollate broth. The minimum inhibiting concentration of a streptothricin preparation (320 units/mg) which was tested in thioglycollate broth No. 1 (7 days after preparation) was 2.65 units/ml as compared to 0.053 units/ml in 0.75% tryptone broth—a ratio of 50:1.

It perhaps should be pointed out that the concentration of sodium thioglycollate in the media tested was 0.5 mg per ml, while the concentrations said by Denkewalter, *et al.*[3] to cause no appreciable destruction of streptomycin ranged from 0.50 to 3.0 mg per ml. Hence remarkable interference with streptomycin activity may occur without destruction of the streptomycin. Since thioglycollates reduce the oxidation reduction potential of a culture medium, could this interference indicate that streptomycin interferes more with aerobic than anaerobic metabolism?

Summary. Under standard test conditions, raising the concentration of tryptone from 0.50% to 1.0%, in a medium containing only tryptone and water, raised the minimum inhibiting concentration of streptomycin from *0.036* units/ml to *0.084* units/ml. The further addition of glucose to the medium increased the minimum inhibiting concentration to a lesser, but nevertheless, definite degree.

The minimum inhibiting concentration of streptomycin in an enriched broth containing sodium glycollate was about 10 times greater than that in 0.75% tryptone. The substitution of sodium thioglycollate for sodium glycollate further greatly increased the MIC. This additional interference by sodium thioglycollate was observed to decrease as the broth aged (and hence as the thioglycollate was oxidized). Since thioglycollate *per se* is said to cause no significant destruction of streptomycin, it is proposed that its interfering action may be due to its role in reducing the oxidation-reduction potential of the medium and that perhaps streptomycin interferes more with the aerobic than anaerobic metabolism of the test organism.

15282

Metabolism of Glycine by the Completely Isolated Mammalian Heart Investigated with Carboxyl-Labeled Glycine.*

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When acetate containing heavy carbon, C^{13}, in the carboxyl position is administered to the completely isolated, working, mammalian heart, the isotope appears in the respiratory CO_2 rapidly and in large amounts, indicating that the 2-carbon chain is readily broken and converted to CO_2, in amounts approximating 20-30% of the total CO_2 output of the heart.[1] In the present experiments, glycine labeled with an excess of C^{13} in the carboxyl carbon, was injected into the blood perfusing the

* Assistance in the preparation of part of these materials was furnished by the personnel of the Work Projects Administration, Official Project No. 165-1-71-440, Sub-project No. 392.

† Present address: Illinois Neuropsychiatric Institute, University of Illinois College of Medicine, Chicago, Ill.

[1] Lorber, V., Lifson, N., Wood, H. G., and Barcroft, J., *Am. J. Physiol.*, 1946, **145**, 557.

TABLE I.
Isotopic Content of Respiratory CO_2 Following Administration of Labeled Glycine to the Isolated Cat Heart.

Exp. No.	Mg of glycine given*	CO_2 collection period in min	mM of carbon collected as respiratory CO_2	% C^{13} in the respiratory CO_2†
1	50	15	0.38	1.07‡
		15	0.53	1.09
		15	0.65	1.10
		17	0.46	1.07
		15	**0.41**	—
		30	1.17	1.10
2	100	30	1.70	1.11‡
		30	1.68	1.07
		30	1.52	1.12
		27	0.70	1.13
3	125	30	1.46	1.08‡
		30	1.42	1.09
		30	1.31	1.12
		34	1.13	1.11

* The glycine was given at the beginning of the second collection period in each experiment.

† $\%\ C^{13} = \dfrac{\text{Moles } C^{13}}{\text{Moles } C^{12} + \text{Moles } C^{13}} \times 100$

‡ Control value prior to injection of glycine. Normal C^{13} content of C from mineral sources is 1.09%.

isolated cat heart in order to determine whether this compound, the amino derivative of acetic acid, is similarly metabolized by cardiac muscle.

Methods. The experiments were performed on cat hearts, of 6 to 7 g weight, isolated in a manner previously described.[2] Respiratory carbon dioxide was collected by passing the ventilating gas through absorption bottles containing carbonate-free sodium hydroxide. Total circulating blood volume was 50 to 60 cc.

Total respiratory carbon dioxide was determined by analysis of the absorption alkali by the Van Slyke manometric method. The C^{13} content of the carbon dioxide for each collection period was measured in the mass spectrometer.[3]

After isolation of the heart was completed, a preliminary control period of 15 to 30 minutes was permitted to elapse, during which time the respiratory carbon dioxide was collected. Following this period, 50 to 125 mg of labelled glycine, dissolved in 0.6 ml of Ringer solution, was injected into the blood, and the carbon dioxide collection was continued, fresh absorption bottles being introduced at 15 to 30 minute intervals. The glycine used was synthesized[4] with 7.59% C^{13} in the carboxyl position.

Results. The results are presented in Table I. A small increase in the % C^{13} in the respiratory CO_2 above the initial control values is noted in each experiment. The irregularities occurring in Exps. 1 and 2, *i.e.*, the low value found in the fourth collection period in 1, and the high value found in the control period in 2, cannot be accounted for, but difficulties in the operation of the mass spectrometer occurring at the time may have been in part responsible. Because of these irrational values, the figures are to be regarded with some reserve. Although if taken at face value the results indicate the breakdown of minute amounts of glycine (about 1 mg) by the heart, the important conclusion to be derived from the data is that the degradation of glycine, under the conditions of our experiments, does not serve as an appreciable source of energy for the heart muscle. This is contrary to the

[2] Lorber, V., *Am. Heart J.*, 1942, **23**, 37.

[3] Nier, A. O., *Rev. Scient. Instruments*, 1940, **2**, 212.

[4] Olsen, N. S., Hemingway, A., and Nier, A. O., *J. Biol. Chem.*, 1943, **148**, 611.

expectation that the high concentrations of glycine maintained, and the low blood carbohydrate values obtaining after 1½ to 2 hours (glucose, 14 mg% at the close of Exp. 2) in the usual experiment of this type, should have favored the metabolism of glycine. Again, with the present results taken at face value, the glycine may be calculated to have yielded only around 0.5% of the total CO_2 output of the heart (on the assumption that both carbons of the molecule have gone to CO_2) as compared with values of 20-30% from acetate,[1] administered in concentrations of the same order of magnitude as those of glycine.

Summary. Glycine, labeled with heavy carbon in the carboxyl position, was administered to the completely isolated, working, cat heart. Isotopic analysis of the collected respiratory CO_2 indicated that if splitting of the 2-carbon chain of glycine with conversion of the carbon to CO_2 was accomplished by the heart muscle, it occurred only to a slight extent.

The authors wish to thank Mr. Charles Stevens, formerly of the Department of Physics, who made the measurements with the mass spectrometer.

15283 P

Blood and Tissue Chemical Studies in Fowl.*

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Normal blood and tissue levels of the usual organic constituents with especial reference to total cholesterol and cholesterol esters and other lipids, were determined as a basis for projected fat metabolism studies. Domestic fowl was the chosen experimental animal because of the known high normal cholesterol production, especially of certified 260 to 320 eggs per year R.O.P. sired hens. Laying hens, after the sixth month, have been found to have a subintimal atherosclerosis of the aorta and coronary arteries, quite similar microscopically to the human type, in half the specimens examined by Dauber.[1] As the age increases, the frequency of gross lesions has been noted to increase.

We have sacrificed 26 barred rocks fed on a standard growing mash (Purina) at ages from 11 weeks through 22 weeks and 26 hens of various standard breeds that had been on a standard laying mash, (Purina). Blood was collected from the severed neck vessels; part was oxalated and part was allowed to clot and the serum expressed. The aorta, heart, and in later studies, about 0.25 g of liver were removed. The gross adipose tissue was dissected off and approximately 0.25 g of cleaned aorta and lean heart muscle and liver were weighed accurately, minced carefully, extracted for 12 hours with 3 to 1 parts of alcohol and ether.

The blood serum was analyzed by Folin's microchemical methods for glucose;[2] non protein nitrogen;[3] and Leiboff and Kahn's method for urea nitrogen;[4] Bloor's original method for cholesterol[5] and Bloor and Knudson's digitonin method[6] for cholesterol esters;

* Supported by a grant from the Medical Research Department, Winthrop Chemical Co. Choline Chloride was generously supplied by Merck & Co.

The generous cooperation of C. W. Carter of the Texas Agricultural Experimental Station, is gratefully acknowledged.

1 Dauber, D. V., *Arch. Path.*, 1944, **38**, 46.

2 Folin, O., *J. Biol. Chem.*, 1929, **82**, 92.

3 Folin and Wu, *J. Biol. Chem.*, 1919, **38**, 81.

4 Leiboff, S. L., and Kahn, B. S., *J. Biol. Chem.*, 1929, **83**, 347.

5 Bloor, W. R., *J. Biol. Chem.*, 1916, **24**, 227.

6 Bloor, W. R., and Knudson, A., *J. Biol. Chem.*, 1916, **27**, 107.

TABLE I.
Blood Chemical Levels in Normal Fowl.

Controls	NPN mg/ 100 ml	Urea mg/ 100 ml	Glucose mg/ 100 ml	Cholesterol mg/ 100 ml	Cholesterol mg/.250 g of aorta	Serum protein g/100 ml	Sex and age
Controls (Y) (26)	38.5	19.6	171.6	217.4	172.7	4.03	Young B. R.
S. D.	±4.7	±2.6	±23.6	±25.0	±33.2	±0.5	11-22 wk old hens
Controls (A) (26)	36.2	19.1	177	248	230	6.1	various
S. D.	±6.4	±3.0	±48.4	±37	±43.2	±1.3	breeds

TABLE II.
Blood and Tissue Chemical Levels in Normal Fowl.

	Blood cholesterol total mg/100 ml	Esters mg/100 ml	Phospholipids mg/100 ml	Tissue cholesterol, total/esters: Aorta mg/250 mg	Heart mg/250 mg	Liver mg/250 mg
Controls (B) (6)	232.	153.		249/	271/	337/
S. D.	±21	±5.6		±29 T / E	±49 T / E	±54 T / E
Controls (C) (26) (4)	271.	179.	9.6	230/176.	288/234.	342/240.
S. D.	±37	±19	2.8	±45/±20	±86/±19	±62/±19

T: total; E: esters.

King's method[7] for total and inorganic phosphorus; and the $CuSO_4$ falling drop method[8] for calculating the total serum protein levels.

The ether alcohol extracts of aortic arches, hearts, and livers were quantitatively analyzed for content of cholesterol, cholesterol esters and the amount in exactly 0.25 g of each tissue was calculated.

The 26 young barred rocks showed normal levels for the usual blood constituents (Table I). The cholesterol levels were relatively low in both sexes from the 11th to 14th weeks and then started to rise, especially in the pullets, seemingly dropped off some at the 16th week as minute egg yolks appeared and then rose slowly. The cholesterol levels in the aortae seemed to follow the blood levels. The blood protein levels were lower than for mammals.

Among the 26 older hens, all showed normal values for the usual blood constituents (Table I), except for the serum proteins which were slightly low, however. The heavy breeds as barred rocks and the high egg producing white leghorns showed the highest levels of lipids in the blood and tissues.

Some further data obtained from control specimens obtained simultaneously with those of the feeding series are given in the Table II.

[7] King, E. J., *J. Biol. Chem.*, 1932, **26**, 292.

[8] Phillips, R. A., *et al.*, U. S. Navy Res. Unit at the Hosp. of the Rockefeller Inst. for M. R., 1943.

15284

Experimental Tubular Nephritis Produced by Safranin O.

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Following the unexpected death of a rabbit 4 days after the intravenous administration of safranin O,‡ autopsy disclosed somewhat enlarged flabby kidneys with swollen yellowish-streaked cortices.§ Microscopic examination of the kidneys revealed severe, essentially selective necrosis of the renal tubular epithelium, involving predominantly the proximal convoluted tubules. The glomeruli appeared uninjured, and there were relatively minor degenerative changes and focal cellular necrobiosis in the loops of Henle and distal convoluted tubules; the collecting tubules appeared normal except for the presence of casts of necrotic material. No noteworthy changes were present in the other organs (brain not examined) except for moderate fatty change and sinusoidal congestion of the liver. The apparent relation of this renal damage to the administration of the dye, and the unexpectedness of this action of safranin, which has been apparently hitherto unreported, prompted a repetition of the procedure for verification and study.

Procedure. Healthy stock rabbits weighing about 2 kg were given a single intravenous administration of safranin O‖ in physiological saline or in aqueous solution, in a dose of 10, 20 or 40 mg per kg. The rabbits were then sacrificed at intervals for gross and microscopic study. In most instances determinations of blood NPN were made; these are recorded in Table I.

Observations. Immediately following injection of the dye, the tissues took on a distinctly pinkish tinge; this was especially noticeable in the ears, conjunctivae, and mucous and serous membranes. It was readily observed in the skin on parting the fur. The kidneys showed a diffuse staining of the cortex and boundary zone within a minute. The dye appeared in the urine within a few hours and was still present at 24 or even 48 hours, depending on the dose.

From Table I, it may be observed that none of 8 rabbits receiving 10 mg per kg showed any significant change in blood NPN. One animal sacrificed at 24 hours showed mild fatty changes and sinusoidal congestion of the liver; except for some excess of granular debris in the tubular lumens and apparent degenerative changes of somewhat questionable significance in the distal portions of the proximal convoluted tubules, the kidneys were not affected at this time. In 2 sacrificed at 48 hours, the greater portion of the renal parenchyma appeared entirely normal; here and there, however, a number of scattered proximal convoluted tubules presented one or several cells showing early but definite coagulation necrobiosis manifested by hypereosinophilia of the cytoplasm, and nuclear pyknosis, lysis and karyorrhexis. Many of these cells were in the process of being extruded into the lumen of the tubule, while adjacent cells appeared entirely normal. In all rabbits, at this dose, sacrificed subsequently, no significant lesions were observed, and any pre-existing damage had been repaired without remaining evidence.

At 20 mg per kg, 4 of 8 rabbits showed a distinct rise in blood NPN at the time of sacrifice. In all 4 animals in which this did

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† With the technical assistance of Elizabeth B. Burkemper and Lt. Charles Boyers.

‡ A basic nuclear stain; one of the azin group of quinone-imine dyes, a mixture of dimethyl and trimethyl diaminophenylphenazonium chloride.

§ The original observation was made by the author in 1942 as an incidental finding in a rabbit submitted for pathological examination by Captain Russel Bowers.

‖ Safranin O, for general staining purposes. Total dye content 85%. C. I. No. 841. National Aniline & Chemical Co., Inc., New York, N. Y.

TABLE I.
Blood NPN of Rabbits Following Intravenous Injection of Safranin O.

Dose (mg/kg)	NPN (mg/100 cc) Before injection	Days after injection 1	2	3	4	5	6	7	9	Fate
10	43	46								Sacrificed 1st day
	49		50							2
	51		59							2
	55			44						3
	47					35				5
	39					40				5
	45		48					56	50	9
	47		42					62	50	9
20	59	60								1
	39	82								1
	40		149							2
	49			46						3
	53		55		54					4
	44					181				5
	37					281				Died 5
	44		52				51			Sacrificed 12
	45		48				65			*
	*		138	98						" 4
40	—									Died 31-47 hr
	—									" ca. 48 "
	59		246							Sacrificed 2nd day
	40			258						" 3
	47			157						" 3
	—									Died 72-96 hr
	30				392					Sacrificed 4th day

* This animal received second injection of 20 mg per kg 12 days after first injection.

not occur, examination of the kidneys revealed relatively mild tubular necrosis, limited to the distal segment of the proximal convoluted tubules and involving apparently only a limited number of nephrons. In those in which the NPN rose, there was extension of the necrosis to the proximal as well as the distal portion of the proximal convoluted tubules, and involvement varying in extent from a moderate proportion to almost all of the nephrons (Fig. 1). In the most severe, there were also distinct degenerative changes and focal necrobiosis of cells in the ascending limb of Henle and in the distal convoluted tubules as well. In all, there was distinct sparing of the nephrons in the inner half of the cortex as compared with the outer half; I have noted this in experimental mercuric poisoning in rabbits also. Similar moderately severe damage was present in another rabbit which had had a previous injection of this same amount 12 days before; the first injection had resulted in but slight rise in NPN while a more marked elevation followed the second injection (Table I). In a dog which also received this amount of safranin, the kidneys presented a similar picture of extensive necrosis of the proximal convoluted cortical tubules.

The dose of 40 mg per kg was administered in 2 separate injections within a period of 2-4 hours, as the injection of this amount as a single dose led to slowing and cessation of respiration. With this amount, 3 of 7 rabbits died at 31-47, 48 and 72-96 hours, respectively. All the others showed marked elevation of NPN at the time of sacrifice. The kidneys of all of the animals of this group showed extensive severe tubular necrosis, even as early as 24 hours (not in Table I), the glomeruli remaining unaffected. It was apparent that the severity of renal damage would unquestionably have led to the death of the sacrificed rabbits as well, had they been permitted to continue. In this, as well as in the other groups, there were no noteworthy findings in the other organs, the brain not being examined, except for mild fatty degeneration and sinusoidal congestion of the liver.

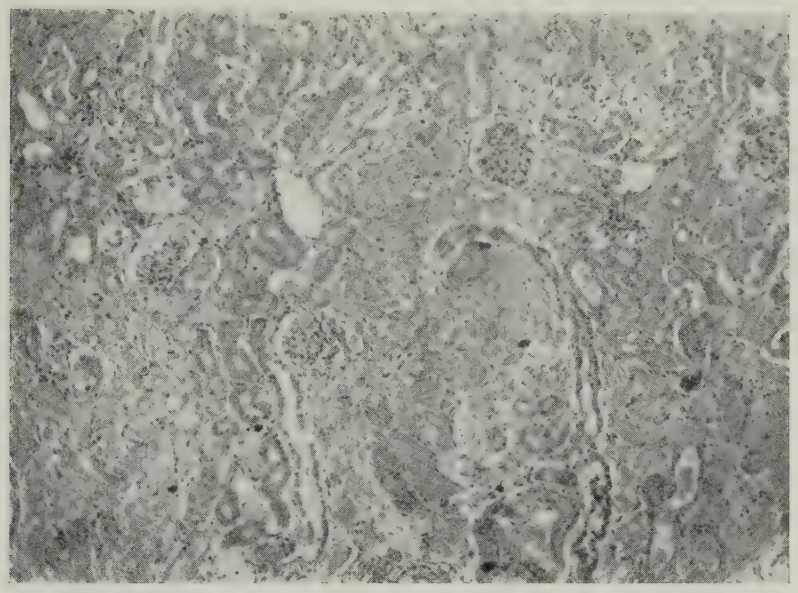

Fig. 1.
Extensive necrosis of epithelium of proximal convoluted tubules in renal cortex of rabbit following intravenous administration of 20 mg per kg of safranin O. On the left a small group of proximal convoluted tubules have escaped damage. The distal tubules are relatively intact except for the presence of casts in the lumen. The glomeruli appear uninjured except for the presence of some granular precipitate in the capsular space of the one at the left.

Discussion. The gross observation of the very rapid deposition of safranin in the kidneys has also been reported by Sheehan[1] who likewise administered this substance to rabbits, but failed to observe any renal or other damage, presumably because of the dose he employed which was apparently 5 mg per kg. Sheehan reported that in unfixed frozen sections of the kidney of rabbits sacrificed within one minute after injection of the dye, heavy staining of intermittent nephrons with the dye could be observed whereas the intervening ones might be altogether uncolored. He also showed that at low rates of renal circulation, the extraction ratio of the kidney for the dye is, for practical purposes, 100% and he has concluded that, whether or not there is glomerular filtration of the dye, a considerable proportion is secreted by the tubules from the peritubular capillaries.

The histologic picture of the renal injury observed in these animals closely resembles, indeed except perhaps for minor detail, is indistinguishable from that caused by mercuric chloride, chromates, uranium, bismuth, and, under certain conditions, cadmium.[2] It is of interest, in regard to biochemical mechanisms of tissue injury, that the pathologic-anatomical effects of heavy metal poisoning should be so closely paralleled by an organic compound such as safranin; in addition, essentially the identical histologic picture of renal tubular injury follows the nephrotoxic action of other organic compounds including oxalates and tartrates, and the dyes, the styrol quinoline dye No. 90, 2(p-acetylamino styryl)6-dimethylamino-quinoline methochloride[3] and acriflavine.[4] The question arises whether similar types of biochemical mechanism of intoxication may not be operative.

[1] Sheehan, H. L., *J. Physiol.*, 1931, **72**, 201.

[2] Ginzler, A. M., *et al., Proc. Am. Assoc. Path. and Bact., Am. J. Path.*, in press.

[3] Sheehan, H. L., *J. Path. and Bact.*, 1932, **35**, 589.

[4] Meleney, F. L., and Zau, Z., *J. A. M. A.*, 1925, **84**, 337.

Considerable evidence indicates that the toxic action of heavy metals may be due to combination with SH enzymes.[5,6] That at least some of the organic compounds mentioned above, specifically safranine, acriflavine and the styrol quinoline compound, may likewise act as inhibitors of enzyme systems and in this way exert their damaging action on the kidneys in a manner paralleling the action of the heavy metals biochemically as well as pathologically, is indicated by Dickens'[7] demonstration of a striking and selective inhibition of the normal aerobic inhibition of fermentation by extremely low concentrations of phenosafranine, the chemical structure of which is closely related to that of safranine, and he has demonstrated a like action of acriflavine as well as a quinoline relative of the styrol quinoline compound No. 90.

Summary. The intravenous administration of the dye, safranin O, to rabbits and dogs produces selective renal tubular necrosis, involving predominantly the proximal convoluted tubules. The histologic picture very closely resembles that observed in heavy metal poisoning.

Safranin O, in a dose of 10 mg per kg, produces minimal histologic injury in the kidney in rabbits, which is readily and rapidly repaired. The intravenous administration of 20 mg per kg is followed by more extensive and severe necrosis of the proximal convoluted cortical tubules, which is, in perhaps half the animals, reflected in elevation of the blood NPN. Increasing the dose to 40 mg per kg results in almost complete necrosis of the renal proximal convoluted tubular epithelium, as well as some damage to the distal tubules; this dose is probably uniformly fatal to rabbits. With all amounts of the substance, noteworthy lesions of other organs than the kidney, in the absence of examination of the brain, appear to be limited to a relatively mild degree of fatty degeneration and sinusoidal congestion of the liver.

The suggestion is made and evidence cited that the nephrotoxic action of safranin, as well as that of certain other nephrotoxic organic compounds, parallels the action of heavy metals biochemically as well as pathologically, and that these organic compounds exert their nephrotoxic effect by acting as inhibitors of vital enzyme systems.

[5] Peters, R. A., and Thompson, R. H. S., *Nature*, 1945, **156**, 616.

[6] Waters, L. L., and Stock, C., *Science*, 1945, **102**, 601.

[7] Dickens, F., *Biochem. J.*, 1936, **30**, 1233.

15285

Potassium Deficiency in the Dog.*

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Recent reports by Lambooy and Nasset[1] and Smith[2,3,4] have indicated that factors, in addition to the better known nutrients, are necessary for optimum growth, prevention of paralysis, maintenance of a healthy skin and prevention of anemia in the dog. We have found a basal ration containing only the 6 water soluble vitamins (thiamin, riboflavin,

* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by a grant from the Wisconsin Alumni Research Foundation.

We are indebted to Merck and Company, Rahway, N. J., for the synthetic vitamins and to Abbott Laboratories, North Chicago, Ill., for haliver oil.

[1] Lambooy, J. P., and Nasset, E. S., *J. Nutrition*, 1943, **26**, 293.

[2] Smith, S. G., *Science*, 1944, **100**, 389.

[3] Smith, S. G., *Am. J. Physiol.*, 1944, **142**, 476.

[4] Smith, S. G., *Am. J. Physiol.*, 1945, **144**, 175.

TABLE I.

	Basal ration (Smith)	Basal ration (modified Street basal)
Sucrose	36%	66%
Casein	40	19
Cottonseed oil	18	11
Cod liver oil	2	0
Salt mixture	4	4
Thiamin	1.4 mg/dog/day	0.1 mg/kg/day
Riboflavin	0.7 " " "	0.1 " " "
Nicotinic acid	6.0 " " "	2.0 " " "
Ca-d-pantothenate	6.0 " " "	0.5 " " "
Pyridoxine	6.0 " " "	0.06 " " "
Choline chloride	100.0 " " "	50.0 " " "
Inositol	100.0 " " "	0 " " "
p-aminobenzoic acid	6.0 " " "	0 " " "
Vit. A	1800 I. U. " "	1800 I. U./dog/day
Vit. B	500 " " "	500 " " "
Vit. E	¾ cc mixed tocopherols/week (Lederle)	0

Smith salt mixture		Salt mixture (Salts IV—Wis.)	
Bone meal, steamed	57.8%	$CaCO_3$	32.34%
Sodium chloride	24.4	K_2HPO_4	34.73
Lime stone	12.2	$CaHPO_4$	5.35
Iron sulfate	3.7	$MgSO_4$	6.67
Magnesium oxide	1.2	NaCl	18.04
Copper sulfate	0.3	$Fe(C_6H_5O_7)_2$	2.37
Manganese sulfate	0.1	KI	0.08
Zinc oxide	0.1	$MnSO_4$	0.36
Cobalt carbonate	0.1	$ZnCl_2$	0.03
Potassium iodide	0.1	$CuSO_4$	0.02

nicotinic acid, pyridoxine, pantothenic acid and choline) together with vitamins A and D to be remarkably satisfactory for growing dogs.[5] In fact, excellent growth and blood regeneration have been obtained even when this ration contained levels of succinylsulfathiazol as high as 4%.[6] Previous results[5] have indicated that these differences are not due to variations in the carbohydrate, fat and protein content of the rations nor to the addition of p-aminobenzoic acid and inositol. However, the low choline level in Smith's ration, even in the presence of high protein, might be a limiting factor. In recent work, we[7] have observed a mortality of 75% in dogs receiving low levels of vitamins in which the choline level was comparable to that fed by Smith (10 mg/kg body weight/day) and autopsy revealed a fatty infiltration of the liver. When the choline level was raised to 50 mg/kg body weight/day, the animals grew normally and remained in good health.

With this information at hand, an experiment was undertaken in which Smith's basal ration (Table I) was duplicated as nearly as possible and fed to a litter of 6 8-week-old pups. For purposes of comparison the composition of the basal ration used in our previous work is also given in Table I. Two of these animals (dogs 1 and 2) received added amounts of choline (50 mg/kg body weight/day) and one dog (dog 6) was fed 7 μg of biotin/kg body weight/day. All animals were fed ad libitum and received an aqueous suspension of the B vitamins twice a week administered by pipette. Three ml blood samples were obtained from the radial vein on the same day every week before the morning feeding, and the hemoglobin

[5] Ruegamer, W. R., Michaud, L., Elvehjem, C. A., and Hart, E. B., *Am. J. Physiol.*, 1945, **145**, 23.

[6] Michaud, L., Maas, A. R., Ruegamer, W. R., and Elvehjem, C. A., PROC. SOC. EXP. BIOL. AND MED., 1944, **56**, 148.

[7] Unpublished data.

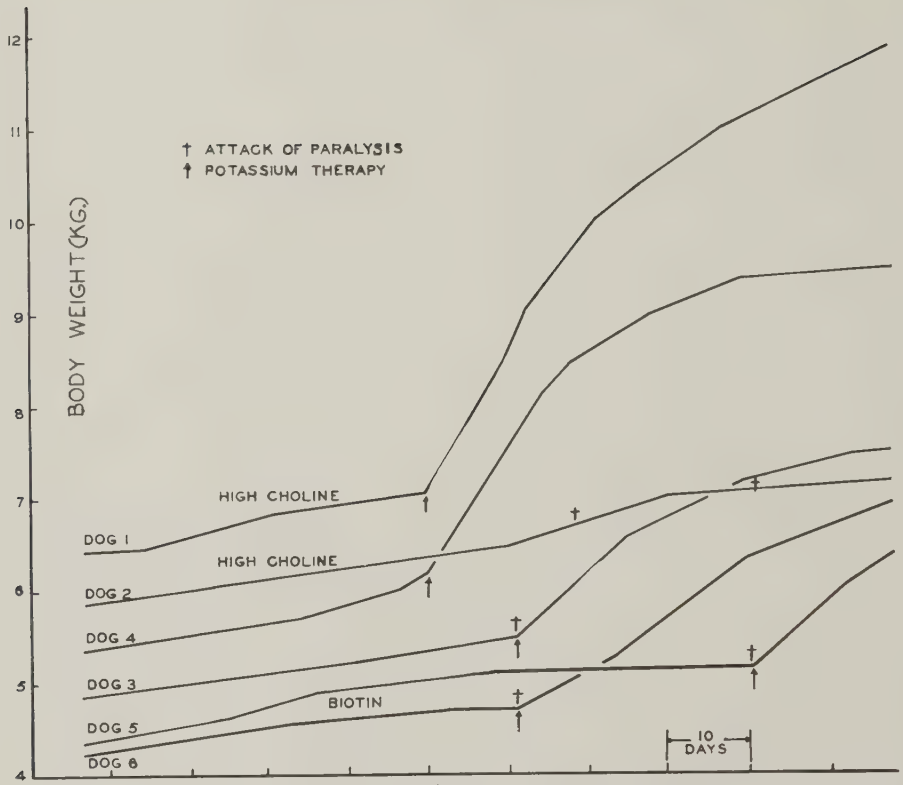

Fig. 1.
The growth response of dogs on the Smith basal ration with and without added potassium.

level determined by the method previously used.[6]

Results. All animals showed poor growth and a plateau in the growth curves was soon evident (Fig. 1). After a few weeks the animals became restless and began to develop symptoms very similar to those described by Smith. At this time, the ration was checked and found to be very low in potassium. In spite of the fact that Smith mentions the administration of potassium chloride to one animal in 100 mg doses every hour for 7 hours without apparent effect, it was decided to try potassium in the case of two of the animals. A single dose of potassium chloride (3 g) was given by capsule to dogs 1 and 4, and the animals continued on the Smith ration but the salt mixture was changed to contain 160 g of KCl/1000 g of salt mixture. An immediate growth response occurred which continued for 30-40 days. One of the animals (dog 1), which received the potassium supplement had been on the high choline level from the start of the experiment and had exhibited no growth until potassium therapy was started.

The remainder of the animals were kept on the Smith basal ration until dog 6, receiving supplements of crystalline biotin, developed a moderate attack of paralysis. The paralysis first started in the muscles of the neck and the animal developed what might be termed a "stiff neck" and was unable to hold its head erect. Several hours later, the animal was given 3 g of potassium chloride by capsule and was placed on the potassium supplemented ration, as in the case of dogs 1 and 4. This animal likewise showed a very marked growth response of approximately 200 g per day, and the paralytic symptoms disappeared completely.

Two days later, dog 3 developed a neck paralysis which grew progressively worse until the animal became severely paralyzed

about 10 hours later. The rear legs were completely paralyzed and the front legs became so weak that the dog was unable to raise itself from the bottom of the cage. At this point, 3 g of potassium chloride was given by capsule and one hour later the animal was able to stand and walk around. This animal was also placed on the potassium supplemented Smith ration and failed to develop any recurrent attacks of paralysis.

Dog 2 (high choline ration) showed 2 attacks of paralysis which were relatively mild and involved only the neck region. The first attack suffered by dog 5 (low choline ration) involved only the neck, but the second attack was quite severe. Three g of potassium chloride was given by capsule and the dog was placed on the potassium supplemented ration. This animal responded well to the potassium therapy and experienced no further transient attacks.

Hemoglobin values ranged between 15 and 16 g per 100 cc of blood just before the animals developed paralysis. Since these animals were only 3½-4 months old at this time, it is possible that a hemo concentration existed as a result of a salt imbalance. After the administration of potassium, the hemoglobin levels dropped to 13-14 g per 100 cc.

As the experiment progressed and before potassium supplements were given, the teeth of all dogs became discolored and the enamel appeared to be eroded. With potassium therapy this condition appeared to improve somewhat but recovery was not complete.

Discussion. When Smith's ration was duplicated as nearly as possible and fed to littermate dogs, all animals ceased to grow and eventually developed paralysis. When potassium therapy was started all dogs demonstrated a rapid growth response of 200-250 g per day and the paralysis disappeared.

It is interesting to note that choline appears to have no effect on the deficiency syndrome observed, since added amounts of choline failed to stimulate growth and prevent the onset of paralysis. It should be pointed out that even though dog 6 received 7 μg of crystalline biotin per kg body weight per day throughout the experiment, this animal suffered attacks of paralysis and failed to grow until potassium therapy was started.

From these data, it would appear improbable that a multiple deficiency developed since the administration of potassium alone brought about an immediate growth response of 200-250 g per day. If there had been a multiple deficiency, it would seem that there should have been an initial response to potassium and that further deficiency would have prevented the animals from making such dramatic weight gains over such an extended period.

The cardiac failure observed so frequently by Smith is another point in favor of an existing potassium deficiency since cardiovascular lesions have frequently been found in rats fed diets deficient in potassium.[8]

In the light of this work, it is difficult to explain the results obtained by Smith with biotin. However in all cases, the biotin seemed to have only a temporary beneficial effect, and the animals soon relapsed into a state of paralysis. Only when the animals were placed on a ration containing 10% yeast which would contain ample amounts of potassium, did the animals recover completely from the paralysis and return to a normal state of health. It is possible, though unlikely, that the administration of potassium increases the intestinal synthesis of biotin, which is actually the curative agent and that our biotin dog did not receive sufficient amounts of crystalline biotin to prevent paralysis.

If the deficiency syndrome observed is due to a potassium deficiency, the paralysis might be explained on the basis of a breakdown in certain enzyme systems. Nachmansohn *et al.*[9] have suggested that potassium seems to be concerned with *in vivo* enzyme systems responsible for phosphorylation. This theory was further substantiated by the work of Boyer *et al.*[10] who found that potassium markedly accelerates the transfer of phosphate from 3-phosphoglycerate to creatine in

[8] McCollum, E. J., Orent-Keiles, E., and Day, H. G., *The Newer Knowledge of Nutrition*, 5th ed., The Macmillan Company, New York, 1939, 203.

[9] Nachmansohn, D., and John, H. M., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 361.

[10] Boyer, P. D., Lardy, H. A., and Phillips, P. H., *J. Biol. Chem.*, 1943, **149**, 529.

homogenized fresh muscle, and that potassium is necessary for the phosphorylation of creatine to accompany pyruvate oxidation by animal tissues. Thus the paralysis may be due to a breakdown in the enzyme system responsible for muscular contraction.

Potassium therapy has been of some value in clinical investigations of certain types of paralysis. Aycock[11] has recently reported a case of familial paralysis in which the administration of potassium brought about a prompt recovery from the paralysis. Likewise, potassium chloride has been found to have a mild effect on the symptoms of myasthenia gravis. Several dog breeders and veterinarians have called our attention to the frequent existence of a paralysis in animals maintained on commercial dog foods. The possibility of a potassium deficiency existing in these rations is being studied at the present time.

Summary. In order to study the paralysis described in dogs by Smith, a ration similar to that described in her work was prepared and fed to growing dogs. When placed on this ration, our animals failed to grow and developed a paralysis which was curable with potassium. Even though Smith has obtained cures with the administration of biotin, one of our animals receiving biotin became paralyzed and responded to potassium therapy.

11 Aycock, W. L., and Foley, G. E., *Am. J. Med. Sc.*, 1945, **210**, 397.

15286 P

Antigenic Relationship of *Shigella dispar*, Types I and II, to *Shigella paradysenteriae*, Boyd Type P143.

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Attempts to classify certain paradysentery-like bacteria have indicated a possible antigenic relationship between *Shigella dispar* and *Shigella paradysenteriae*, type P143.[1] In particular, a culture (No. 2-193) isolated by Major William H. Ewing, Sanitary Corps, United States Army, has been classified on various grounds as *Sh. paradysenteriae*, type P143,[2] *Sh. alkalescens*,[3,4] and *Sh. dispar*.[2] The present investigation was undertaken to attempt to clarify the relationship between *Sh. dispar* and type P143.

Four serotypes and several subtypes of *Sh. dispar* were previously reported.[5] Representative strains of these were employed. Type P143 was secured through the courtesy of Dr. K. M. Wheeler of the Connecticut Public Health Laboratories as his culture No. 573.[6] Antiserums were prepared by immunizing rabbits with living vaccines of this and of the type and subtype strains of *Sh. dispar*.

Cross agglutination tests indicated the existence of antigens common to type P143 and *Sh. dispar*, types I and II. This was confirmed by reciprocal adsorption of the cross-reacting serums, as shown in Table I. From these data were derived the partial antigenic formulae listed in Table II. The relationship between *Sh. dispar*, types I and II, and *Sh. paradysenteriae*, type P143, is apparent. While antigens *B*, *C* and *D* are apparently minor factors in P143, nevertheless, they appear to account for the strong agglutination (to titers of 1280 or 2560) of types I and II in antiserum P143.

Culture 2-193 is biochemically like *Sh.*

1 Boyd, J. S. K., *J. Hyg.*, 1938, **38**, 477.

2 Ewing, W. H., personal communication.

3 Neter, E., Proc. Soc. Exp. Biol. and Med., 1944, **57**, 200.

4 Neter, E., *J. Imm.*, 1945, **51**, 151.

5 Carpenter, P. L., *J. Bact.*, 1944, **47**, 419.

6 Wheeler, K. M., *J. Imm.*, 1944, **48**, 87.

TABLE I.
Cross-agglutination and Reciprocal Adsorption of *Sh. dispar*, Types I and II, and *Sh. paradysenteriae*, Type P143.

Serum	Adsorbed with strain	Agglutinative titer with *Sh. dispar* serotype I (171)	IIa (167)	IIb (205)	IIc (231)	*Sh. para.* P143
171	Unads.	5120*	640	1280	1280	80
	167	5120	0†	0	0	80
	205	5120	0	0	0	80
	231	5120	0	0	0	40
	P143	2560	640	640	1280	0
167	Unads.	640	81920	40960	40960	640
	171	0	20480	5120	10240	160
	205	0	2560	0	2560	320
	231	0	0	0	0	0
	P143	320	20480	20480	20480	0
205	Unads.	320	10240	10240	10240	20
	171	0	10240	5120	10240	20
	167	0	0	0	0	0
	231	0	0	0	0	0
	P143	160	10240	5120	5120	0
231	Unads.	1280	10240	10240	10240	320
	171	0	10240	10240	5120	160
	167	0	0	0	160	0
	205	0	80	0	2560	40
	P143	1280	10240	10240	10240	0
P143	Unads.	1280	2560	2560	2560	10240
	171	0	320	1280	640	2560
	167	320	0	0	0	2560
	205	80	160	0	20	2560
	231	160	0	0	0	2560

* Titers are expressed as reciprocals of the highest dilutions giving any agglutination after 15-18 hours incubation at 55°C.

† *0* indicates no agglutination in 1:40 dilution.

TABLE II.
Antigens of *Sh. dispar*, Types I and II, and *Sh. paradysenteriae*, Type P143.

Culture No.	Type	Antigens
171	*Sh. dispar* I	A.C.
167	*Sh. dispar* IIa	AB.D.
205	*Sh. dispar* IIb	AB.
231	*Sh. dispar* IIc	AB.DE.
P143	*Sh. paradysenteriae* P143	BCD.F.
2-193		AB.

alkalescens. Neter[3,4] has recently so classified this strain. However, he stated that it does not share any major antigens with *Sh. alkalescens* types I, II or IV, and established a separate serotype (III) for it. He noted that this strain is strongly agglutinated by antiserum P143. Wheeler *et al.*[7] reported strong cross reactions between culture 2-193 (Wheeler's No. 2372) and type P143, and postulated a common antigen distinct from the specific P143 antigen. They also noted very close relationship between 2-193 and *Sh. dispar*, type II.

In the present investigation, strain 2-193 agglutinated in 1:2560 dilution of antiserum P143 (homologous titer 1:10,240). Conversely, P143 agglutinated to a titer of 1:320 in an antiserum for 2-193 (supplied by Dr. Wheeler) whose homologous titer was 1:20,480. By use of monovalent serums prepared by suitable adsorption (Table II), major antigens *A* and *B* were found in strain 2-193. It is therefore antigenically a typical *Sh. dispar*, type IIb. It is to be noted that fraction *B* is the antigen common to 2-193 and P143. Furthermore, culture 2-193 agglutinated to titer in antiserum 205 (type IIb) and almost completely adsorbed the latter (titer reduced from 20,480 to 160).

[7] Wheeler, K. M., Stuart, C. A., and Ewing, W. H., *J. Bact.*, 1946, in press.

Likewise, antiserum 2-193 agglutinated strain 205 to titer, and when adsorbed with 205 had a residual homologous titer of only 40.

It thus appears that antiserum P143 may be rendered more nearly monovalent by adsorption with *Sh. dispar,* type I and IIa or IIc. This procedure would prevent confusion in classification of cultures such as 2-193.

15287

Immunity Following Para-Aminobenzoic Acid Therapy in Experimental Tsutsugamushi Disease (Scrub Typhus).

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(Introduced by S. Bayne-Jones.)

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Two recent communications[1,2] from this laboratory have dealt with the therapeutic effectiveness of para-aminobenzoic acid (PABA) in the treatment of experimental tsutsugamushi disease. The beneficial effect of PABA and especially that of the sodium salt of the acid (NaPAB) was demonstrated against the Ceylon, Calcutta, and Karp strains of *R. orientalis*[1,2] and later against an Imphal strain.[3] The animals used in these experiments were 2 species of desert rodents, *Gerbillus pyramidum* and *Gerbillus gerbillus,* which are abundant in Egypt.[4] The susceptibility of gerbilles to these strains of *R. orientalis* and the manifestations of the disease in them have been detailed elsewhere.[5]

Para-aminobenzoic acid has also been shown experimentally to have a beneficial therapeutic effect on murine[6-9] and epidemic typhus,[7] and on Rocky Mountain spotted fever.[10,11] Clinical trial of PABA has demonstrated it to be of value in the treatment of louse-borne typhus fever.[12] There has also been recently reported a case of Rocky Mountain spotted fever which received PABA with apparent benefit.[13]

Thus, it has been shown by several investigators that PABA has an effect on several rickettsial agents. The mode of action of PABA on these organisms is of great interest since they are obligate intracellular parasites. Various studies carried out in developing chick embryos indicate that PABA is rickettsiostatic rather than rickettsiocidal.[7-10] Furthermore, in PABA treated human cases of epidemic typhus fever and the case of Rocky Mountain spotted fever there was no apparent disturbance of the antibody dynamics as judged from findings in Weil-Felix and complement-fixation

1 Snyder, J. C., and Zarafonetis, C. J. D., PROC. SOC. EXP. BIOL. AND MED., 1945, **60**, 115.

2 Murray, E. S., Zarafonetis, C. J. D., and Snyder, J. C., PROC. SOC. EXP. BIOL. AND MED., 1945, **60**, 80.

3 Unpublished observations of the authors.

4 Snyder, J. C., Zarafonetis, C. J. D., and Lui, W. T., PROC. SOC. EXP. BIOL. AND MED., 1945, **59**, 110.

5 Zarafonetis, C. J. D., PROC. SOC. EXP. BIOL. AND MED., 1945, **59**, 113.

6 Snyder, J. C., Maier, J., and Anderson, C. R., Report to the Division of Medical Sciences, National Research Council, Washington, D. C., December 26, 1942.

7 Hamilton, H. L., Plotz, H., and Smadel, J. E., PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 255.

8 Greiff, D., Pinkerton, H., and Moragues, V., *J. Exp. Med.,* 1944, **80**, 561.

9 Greiff, D., and Pinkerton, H., *J. Exp. Med.,* 1945, **82**, 193.

10 Hamilton, H. L., PROC. SOC. EXP. BIOL. AND MED., 1945, **59**, 220.

11 Anigstein, L., and Bader, M. N., *Science,* 1945, **101**, 591.

12 Yeomans, A., Snyder, J. C., Murray, E. S., Zarafonetis, C. J. D., Ecke, R. S., *J. A. M. A.,* 1944, **126**, 349.

13 Rose, H. M., Duane, R. B., and Fischel, E. E., *J. A. M. A.,* 1945, **129**, 1160.

tests.[12,13] These observations support the previously advanced hypothesis that PABA inhibits the multiplication of rickettsiae inside the cells, thereby permitting the immunity mechanisms of the body to dispose of them.[12] The treatment of these rickettsial infections with PABA, therefore, should not interfere with the development of immunity to them. This has been shown to be true in guinea pig experiments with Rocky Mountain spotted fever.[13] In order to obtain additional data, however, immunity tests were carried out in gerbilles which had survived inoculation with scrub typhus with the aid of PABA therapy. The purpose of this report is to give the results of these tests.

Immunity Tests. Six to 9 months after the PABA therapy experiments were concluded, there were 69 treated gerbilles available for immunity tests. Of these, 24 were from the Ceylon strain experiments, 4 from the Calcutta, 32 from the Karp, and 9 from the Imphal strain experiment. The gerbilles had gained weight and appeared healthy at the time of testing for immunity to their respective homologous strains.

The test inocula consisted of peritoneal washings from one or more gerbilles which had been infected with the strain desired. Three cubic centimeters of sterile physiological saline solution were used to make the peritoneal washings, and, from this, serial ten-fold dilutions were made. Each gerbille was inoculated intraperitoneally with 1 cc of the 10^{-2} dilution of homologous strain peritoneal washings. Simultaneously, each strain inoculum was titrated in normal gerbilles in dilutions from 10^{-1} through 10^{-6}. The animals were then observed for 21 days, at which time the experiment was arbitrarily closed.

There was but one death in the 69 gerbilles tested, though the animals had received from 2,140 to over 3,600 lethal doses of infectious material. The single death occurred in a gerbille of the Karp strain group. It died on the 11th post-inoculation day and autopsy was not at all suggestive of scrub typhus. The liver and spleen appeared normal and there was no pleural effusion. Large numbers of bacteria were seen in smears of the peritoneal exudate.

It appears, then, that these gerbilles which had been infected with scrub typhus and survived through treatment with PABA developed a strong immunity to the homologous strain of *R. orientalis*. It should be noted, however, that Fox and Peterson have found that *R. orientalis* persist up to a year or more in mice which survived either as a result of methylene blue treatment or as a result of infection by subcutaneous inoculation.[14] The presence of active organisms in the cells of an infected animal might well give rise to "interference phenomenon" so that subsequent survival to re-inoculation might not represent immunity in the usual sense of the term. The presence of surviving rickettsiae from primary inoculation was not investigated in the gerbilles used in these tests. However, the fact that the gerbilles resisted challenge indicates that infection was established on primary inoculation in spite of PABA treatment. If immunity developed in these gerbilles and is the reason for their survival, then, the effect of PABA was to inhibit the rickettsiae permitting the mechanisms of immunity to develop. Should living *R. orientalis* be demonstrated in animals surviving infection with the aid of PABA treatment as it has been in the case of methylene blue treated mice, the conclusion would again be that the effect of PABA was rickettsiostatic rather than rickettsiocidal.

Cross-immunity Tests. Cross-immunity between various strains of *R. orientalis* has been demonstrated on several occasions. The literature on this subject has been recently reviewed by Blake, *et al.*[15] who also investigated cross-immunity with other strains. Additional strains have since been tested by other workers.[16] However, cross-immunity has not been described for the 4 strains involved in these PABA studies. Therefore, upon the completion of the immunity tests

[14] Fox, J., and Peterson, O., personal communication.

[15] Blake, F. G., Maxcy, K. F., Sadusk, J. F., Jr., Kohls, G. M., and Bell, E. J., *Am. J. Hyg.*, 1945, **41**, 243.

[16] Kohls, G. M., Armbrust, C. A., Irons, E. N., and Philip, C. B., *Am. J. Hyg.*, 1945, **41**, 374.

of the PABA treated gerbilles with their respective homologous strains of *R. orientalis,* cross-immunity tests were performed with heterologous strains. Inocula were prepared and administered in the same manner as described for the immunity tests. Simultaneous titrations of the infectious material were made in normal gerbilles with dilutions of 10^{-1} through 10^{-8}. The test period of 21 days remained the same.

Of the 24 gerbilles immune to the Ceylon strain, 9 were tested with the Karp strain, 8 with the Calcutta, and 7 with the Imphal strain. One death occurred in the group challenged with the Calcutta strain, 15 days after the inoculation. This gerbille showed evidence of marked weight loss, but the autopsy was otherwise negative. No rickettsiae were seen in peritoneal smears.

Three gerbilles immune to the Calcutta strain were challenged with Karp strain inoculum and survived.

The 30 Karp-immune gerbilles were divided into 3 groups: 9 were inoculated with the Ceylon strain, 10 with the Calcutta strain, and 11 with Imphal strain infectious material. There were no deaths among these gerbilles.

The 9 gerbilles immune to the Imphal strain were divided into 3 groups of 3 which were tested with the Ceylon, Calcutta, and Karp strains, respectively. All of the gerbilles survived.

The gerbilles which were tested with the Karp strain received 316,000 lethal doses; those tested with the Calcutta strain received 31,600 lethal doses; while those receiving Ceylon and Imphal infectious material survived 2,140 and 3,160 lethal doses, respectively. Thus, it is apparent that a solid immunity against heterologous strains of *R. orientalis* was present in these gerbilles. It is possible, of course, "interference phenomenon" may have exerted an effect of undetermined degree in these tests.

Summary. Gerbilles which were infected with tsutsugamushi disease and survived through treatment with PABA were tested 6 to 9 months later and found to be immune to homologous strains of *R. orientalis.* This is interpreted as evidence that the mode of action of para-aminobenzoic acid in scrub typhus is rickettsiostatic.

Cross-immunity tests revealed that these gerbilles were also immune to heterologous strains of *R. orientalis.*

The technical assistance of Cpl. George Tasker and Sgt. Robert Stearman is gratefully acknowledged.

15288 P

Natural Occurrence of an "Induced" Antigen in Salmonella Cultures.*

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It is known[1-6] that H antigens of *Salmonella* can be markedly changed by cultivating the organisms in serum containing agglutinins for the flagellar components. Excepting *S. abortus-equi*[4] and *S. paratyphi A*,[5] all antigens so obtained have had little or no relationship to naturally occurring H antigens of the genus, and have been referred to as induced

* The investigation reported in this paper is in connection with a project of the Kentucky Agricultural Experiment Station and is published by permission of the Director.

1 Kauffmann, F., *Z. Hyg.,* 1936, **119**, 104.

2 Gnosspelius, A., *Z. Hyg.,* 1939, **121**, 529.

3 Kauffmann, F., and Tesdal, M., *Z. Hyg.,* 1937, **120**, 168.

4 Edwards, P. R., and Bruner, D. W., *J. Bact.,* 1939, **38**, 63.

5 Bruner, D. W., and Edwards, P. R., *J. Bact.,* 1941, **42**, 467.

6 Edwards, P. R., and Bruner, D. W., *J. Bact.,* 1942, **44**, 289.

or artificial forms. Whether these are minor components of the flagellar complex brought into prominence, or artifacts produced by action of the serum, has been the subject of some discussion. Hitherto, such forms have not been recognized in nature, although Eriksson and Malmström[7] found agglutinins for an induced phase of *S. newport* in the serum of a patient infected with that type. The present note records the occurrence in nature of antigens apparently identical with those obtained by cultivation of *Salmonella* in agglutinating serum.

Since April, 1945, 14 cultures received from Connecticut, Maryland, Florida, Illinois, California and Uruguay, were apparently monophasic variants of *S. minnesota,* which had the formula XXI, XXVI:b. Phase 2 (e,n,x, . . .) could not be demonstrated. When stabbed into semi-solid agar containing b serum, the cultures were either confined to the site of inoculation or produced one or 2 small bulbs of spreading growth which yielded a form flocculated no longer by serums for the known H antigens of the genus but by serums for certain induced antigens. By continued transfer in b serum, such forms were obtained from 13 of the cultures. While this work was in progress, 2 cultures isolated from sewage by Mr. A. A. Hajna were received. These cultures also had O antigens XXI, XXVI and gave H reactions similar to those cited above. Serums were prepared from one of the induced forms of the XXI, XXVI:b cultures and one of the sewage cultures. Reciprocal agglutination and absorption tests indicated that the forms obtained by growth in b serum were identical with the cultures from sewage. A symbol (z_{33}) was assigned the H antigens of these forms. By cultivation of the z_{33} form of the XXI, XXVI:b cultures in z_{33} serum, e,n,x,. . . phases were obtained from 5 of them. One reverted to b. The 2 sewage cultures (XXI, XXVI:z_{33}) were cultivated in z_{33} serum and an e,n,x. . . phase was obtained from one. The z_{33} and e,n,x. . . forms are quite resistant to change. As yet a change from b directly to e,n,x. . . has not been accomplished. The changes brought about in the 2 naturally occurring forms may be summarized as follows:

XXI, XXVI:b $\rightleftharpoons$ XXI, XXVI:z_{33} $\rightarrow$ XXI, XXVI:e,n,x. . .

XXI, XXVI:z_{33} $\rightarrow$ XXI, XXVI:e,n,x. . .

Since so little is known concerning phylogeny and evolution in the *Salmonella,* it is difficult to interpret these results. A complex parent containing b, e,n,x. . . and z_{33} could be postulated for *S. minnesota* (XXI, XXVI:b-e,n,x. . .) and the XXI, XXVI:b and XXI, XXVI:z_{33} forms. On the contrary it is possible that the XXI, XXVI:b cultures are loss variants of *S. minnesota* which give rise to XXI, XXVI:z_{33} forms under certain environments. The fact that in the progression b $\rightarrow$ z_{33} $\rightarrow$ e,n,x. . . only z_{33} gave rise to the e,n,x. . . phase which would normally be expected in these cultures, indicates that z_{33} is not an artifact or denatured antigen, but a minor constituent brought into prominence. Other antigens hitherto observed only in cultures exposed to agglutinating serum probably also occur in nature. These observations agree with those of Monteverde and Leiguarda[8] and of Edwards[9] that *S. ballerup* (XXIX[Vi]:z_{14}) can be changed to a form resembling *S. hormaechei* (XXIX [Vi]:z_{30},z_{31}). They are further supported by unpublished results of the writers, who succeeded in changing all of 9 cultures of *S. simsbury* (I, III, XIX: z_{27}) to forms serologically indistinguishable from *S. senftenberg* (I, III, XIX: g,s,t. . .). It is not yet possible to say whether the transformability of these apparently distinct types means that serologically divergent strains of *Salmonella* may arise by processes analogous to induced variation or whether these apparently distinct types are merely loss variants of complex parents. In the transformation of *S. salinatis* (IV, XII: d,e,h-d,e,n,z_{15}) to *S. sandiego* (IV, XII: e,h-e,n,z_{15})[6] and in the appearance of XXIX[Vi]:z_{30} and XXIX[Vi]:z_{31} variants of *S. hormaechei,*[9] only loss variation seems operative. In any event, a re-examination of some of the apparently distinct *Salmonella* types is indicated.

[7] Eriksson, E., and Malmström, F., *Z. Hyg.,* 1939, **121**, 629.

[8] Monteverde, J. J., and Leiguarda, R. H., *Nature,* 1944, **153**, 589.

[9] Edwards, P. R., *J. Bact.,* in press.

15289 P

Production of Staphylococcal Enterotoxin in Chemically Defined Media.*

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Production of staphylococcal enterotoxin in synthetic media has not been previously reported, although Favorite and Hammon[1] and Hammon[2] used successfully a casein hydrolysate medium. The present is a report of enterotoxin production in synthetic media modified from those used by Gladstone[3] in studies of the hemolysins.

Four enterotoxigenic strains (Nos. 8, 147, 161 and 196) and one non-enterotoxic strain (No. 184) of staphylococci were used in these experiments. All strains are alpha-hemolytic and strains No. 184 and 196 produce potent beta lysin.

The compositions of 3 basic media used in these experiments are given in Table I. Media were adjusted to pH 7.6, dispensed into nursing bottles or flasks and sterilized in the autoclave. Inoculations were made with 0.1 ml of a 1:1000 dilution (in phosphate buffer) of culture in synthetic medium.

Cultures were grown at 37°C in an atmosphere of 20% CO_2. During incubation they were rotated continuously on a vertical wheel. Centrifugates of 3 to 7 day cultures were tested for enterotoxin and hemolysins.

Enterotoxin was assayed by the monkey *(Macacca mulatta)* feeding and intravenous cat tests.[4,5] Untreated centrifugates were fed to monkeys; preparations heated for 30-35 minutes in a boiling water bath were injected into cats. Vomiting was used to indicate a positive reaction. Alpha-hemolysin titers are expressed in terms of the M.H.D. (minimal hemolytic dose) of toxin.[6] Beta-lysin tests were made against sheep erythrocytes and

TABLE I.
Composition of Basic Media.

Amino acids	Concentration	Medium 1	Medium 2	Medium 3
l (+) arginine HCl	M/110	+	+	+
l-cystine	M/10,000	+	+	+
dl-alanine	M/1500	+	+	
glycine	M/198	+	+	
dl-phenylalanine	M/4000	+	+	
dl-valine	M/1500	+	+	
l-aspartic acid	M/1500	+		
d-glutamic acid	M/1500	+		
l-histidine 2HCl	M/4000	+		
l-hydroxyproline	M/1500	+		
l-leucine	M/1500	+		
d-lysine 2HCl	M/4000	+		
dl-methionine	M/4000	+		
l-tryptophane	M/20,000	+		
l-tyrosine	M/4000	+		

The media also contained M/6000 $MgSO_4$; M/20,000 $FeSO_4.(NH_4)_2SO_4.6H_2O$; M/30 KH_2PO_4; M/37 NaOH; M/80 glucose; 1.23 μg/ml nicotinic acid; 0.0337 μg/ml thiamine HCl; 1.0 μg/ml calcium pantothenate (omitted from Media No. 1 and No. 2 in early tests).

* This work was supported by a grant from the National Canners Association.

1 Favorite, G. O., and Hammon, W. McD., *J. Bact.*, 1941, **41**, 305.

2 Hammon, W. McD., *Am. J. Public Health*, 1941, **31**, 1191.

3 Gladstone, G. P., *Brit. J. Exp. Path.*, 1938, **19**, 208.

4 Davison, E., Dack, G. M., and Cary, W. E., *J. Infect. Dis.*, 1938, **62**, 219.

5 Dack, G. M., 1943, *Food Poisoning*, University of Chicago Press, Chicago.

6 Dolman, C. E., and Kitching, J. S., *J. Path. and Bact.*, 1935, **41**, 137.

TABLE II.
Production of Staphylococcal Enterotoxin and Alpha Hemolysin in Chemically Defined Media.

Medium		Staphylococcal enterotoxin: Monkey feeding test			Staphylococcal enterotoxin: Intravenous cat test			Alpha hemolysin
Basic*	Modification	Dosage (ml per monkey)	Reaction pos.	Reaction neg.	Dosage (ml per kg body wt)	Reaction pos.	Reaction neg.	M.H.D. per ml
No. 1		50	0	5	1.0-2.0	10	3	4-16
	+ proline M/690	50	0	4	1.0	2	2	64-128
	+ " M/280	50	6	3	1.0	6	3	32-64
	+ " M/280 — aspartic acid glutamic " histidine	50	1	0				4-16
No. 2		40-100	2	2	2.0	1	2	32
	+ proline M/690	50	0	2	2.0	2	2	16-32
	+ glutamic acid M/280	40-100	1	2	2.0	2	1	64
	+ hydroxyproline M/280	40-100	1	3	2.0	2	1	16-32
No. 3		55-100	0	3	2.0	0	4	<4
	+ $(NH_4)_2SO_4$ M/250	50-100	0	3	2.0	1	3	4-16
	+ glycine M/198	40-100	0	3	2.0-3.0	3	1	8

* Numbers correspond to Table I.

were read after incubation for one hour at 37°C followed by one hour in an ice water bath.

Results of tests for enterotoxin and alpha-lysin are shown in Table II. Alpha hemolysin production and vomiting reactions in cats were obtained with cultures grown in all media except Medium No. 3. In the latter medium negative results may be correlated with nitrogen depletion, since addition of $(NH_4)_2SO_4$ or glycine increased growth hemolysin titers and enterotoxic reactions. Four cats failed to react to centrifugates of the nonenterotoxic strain grown in Medium No. 1. Monkeys reacted irregularly to centrifugates of cultures grown in the more complex media. The positive tests are significant since they demonstrate toxicity following feeding. Beta-lysin was detected in centrifugates of beta-positive strains in titers ranging from 8 to 256 M.H.D. (for sheep r.b.c.) per ml.

In general growth and hemolysin production decreased with simplification of the media. Qualitative tests suggest a similar decrease in enterotoxin production.

Summary. Production of staphylococcal enterotoxin in chemically defined media is reported. These experiments have not demonstrated specific requirements for enterotoxin production.

15290

Bone Tumors in Fowls Injected Intravenously with Causative Agent of Rous Sarcoma.

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It is well known that intravenous injections of the causative agent of filterable fowl sarcoma into fowls never produce widespread sarcomatosis and, in the absence of a primary tumor, seldom cause solitary sarcomas in the visceral organs.[1-7] The agent introduced into the blood stream rapidly disappears from the circulation, probably being taken up by the cells of the reticulo-endothelial system.[8] There is general belief that the sarcoma producing agent injected into the circulation initiates a new growth only at points of vascular injury, *i.e.* where the agent comes into contact with the connective tissue cells. This can take place in the ovary as a sequence of frequent injuries to the vessels occurring in this organ during the process of ovulation.[1] Experimentally the agent can be localized by thermo-cauterisation of the skin,[3] by injections of histamine,[6] by puncture of the vessel,[2] by inducing the development of reaction tissue,[4] etc.

We wish to report in the following our experiments on intravenous injections of the causative agent of Rous sarcoma into chicks, the results of which deviate considerably from previous observations.

In the present study we used White Leghorn chicks of both sexes from the same farm. As a source of the causative agent cultures of filterable fowl sarcoma (strain Rous-Fischer) were employed. This tumor has been maintained in this laboratory for the past 10 years by serial passages *in vitro*. The following design of experiment was adopted: fragments of a Rous cell colony were planted into a Carrel flask containing chicken plasma diluted with Tyrode solution in the proportions of 1:2. After cultivation for 1-3 weeks the flasks were overlaid with Tyrode solution and placed in the incubator for 30 minutes; the supernatant fluid was pipetted off and centrifuged 3 times at 3000 r.p.m. for 15 minutes each time.

Twelve chicks, aged 4 to 10 weeks, in 4 experimental groups were injected with 1 ml of this fluid into the wing vein, 2 or 3 injections being given at weekly intervals to each chick. The fowls succumbed in 21 to 42 days after the initial injection. Necropsy showed new growths in the liver, lungs, heart and kidneys in 9 of the 12 chicks. The tumors in the viscera were always associated with a tumor at the site of the inoculation.

The outstanding feature observed in all chicks was the following: 11 of 12 chicks injected with sarcoma agent displayed most extensive neoplastic bone changes, involving one or several bones (sternum, femur, tibia, tarso-metatarsus, radius, ulna, humerus). Histologically, the tumors were found to be typical osteoid sarcoma destroying the bone and invading the nearby muscles.

The appearance of a general bone neoplasia in a series of animals injected intravenously with Rous agent is noteworthy. This behaviour of Rous agent injected into the blood stream has never been described and in the 10 years during which we have worked with our strain in this laboratory this is the first time that bone tumors appeared either after

1 Rous, P., Murphy, J. B., and Tytler, W. H., *J. A. M. A.*, 1912, **58**, 1751.

2 Pentimalli, F., *Z. f. Krebsforsch*, 1924, **22**, 74.

3 Findlay, G. M., *J. Path. and Bact.*, 1928, **31**, 633.

4 Mackenzie, R. D., and Sturm, E., *J. Exp. Med.*, 1928, **47**, 345.

5 Cramer, W., *Ninth Scientific Report, Imperial Cancer Research Fund*, London, 1930, 21.

6 Doerr, R., Bleyer, L., and Schmidt, G. W., *Z. f. Krebsforsch*, 1932, **36**, 256.

7 Sittenfield, M. J., Johnson, B., and Jobling, J. W., *Am. J. Cancer*, 1932, **16**, 345.

8 Mellanby, E., *J. Path. and Bact.*, 1938, **47**, 47.

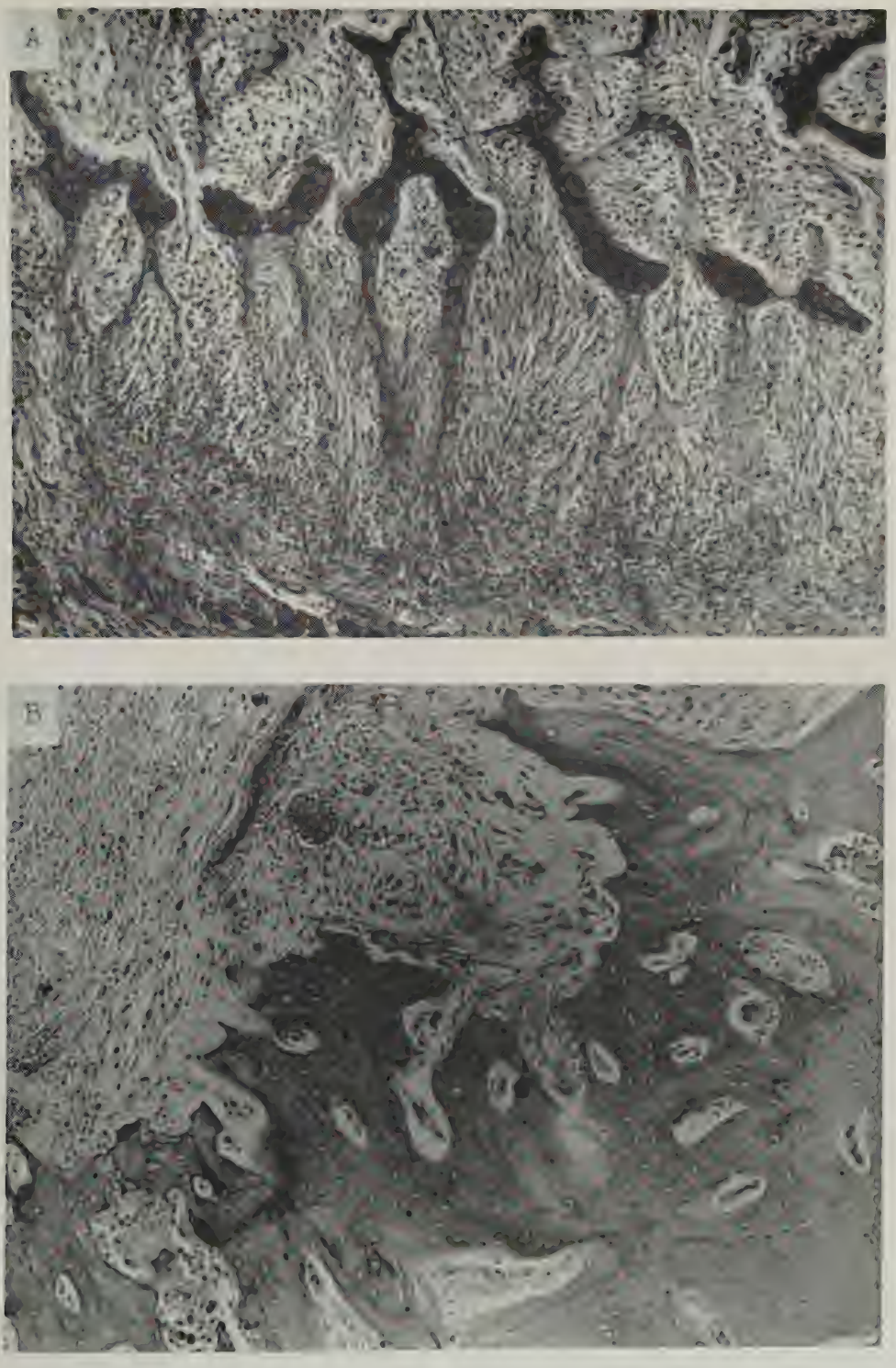

Fig. 1.
a. Chicken N. 3447. Bone tumor. Hematoxylin-Eosin. Mag. X 145.
b. Chicken N. 3449. Bone tumor. Hematoxylin-Eosin. Mag. X 135.

intramuscular or intravenous injections. The cause of this unusual effect of the intravenous inoculation of Rous agent in the experiments reported is entirely obscure. We do not know whether it is to be sought in the particular manner of applying the agent (repeated intravenous injections) or whether we are dealing with a real modification of the agent, which took place spontaneously or resulted from long passage *in vitro*. All these possibilities deserve consideration.

During the preparation of this note a paper of Shrigley, Greene and Duran-Reynolds[9] appeared. The authors observed that the Rous sarcoma agent after remaining in the anterior chamber of the eye of the guinea pig acquired the ability to produce periosteal tumors in chicks. To judge by the description and the photomicrograph of these growths, they are very similar to those here reported. The acquisition of a new tissue affinity was ascribed to modification of the agent caused by its sojourn in an unnatural environment.

Summary. The appearance of an osteoid sarcoma in a series of chickens injected intravenously with the causative agent of Rous sarcoma derived from sarcoma cell cultures, is reported.

[9] Shrigley, E. W., Greene, H. S. W., and Duran-Reynals, F., *Cancer Research*, 1945, **5**, 356.

15291 P

Development of Tolerance to Typhoid Bacterial Pyrogen and its Abolition by Reticulo-Endothelial Blockade.*

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Patients who are being given fever therapy by means of intravenous injections of typhoid vaccine exhibit a decreasing reactivity, and must be given larger and larger doses of the vaccine in order to develop similar fevers at successive treatments. After several injections some individuals may require doses as large as 250 ml of typhoid vaccine in a single day.[1] The mechanism of this remarkable tolerance has not been explained. The present report deals with a study of the phenomenon in rabbits.

The rabbits used were males, of mixed breed, weighing 2 to 3 kg. During test periods they were placed in wooden stalls, and held by head boards. Rectal temperatures were taken at 30-minute intervals, through openings in the floors of the stalls. Observations were never continued for more than 7 hours after giving vaccine, to prevent undue fatigue of the animals. The vaccine used contained approximately one billion killed *E. typhosa* per ml.† The dose was 1 ml of a 1:8 dilution in physiologic salt solution. The agent used for blockade of the reticulo-endothelial system was colloidal thorium dioxide (Thorotrast-Heyden Co.); 9 ml was injected intravenously.

The febrile responses to daily injections of the same dose of vaccine were recorded on 40 rabbits during periods of from 8 to 45 days. The first injection of vaccine always caused a rise in body temperature of 4-5°F, and some fever persisted throughout the 7-hour period of observation. The 2nd and 3rd injections generally caused almost as much fever as the first, but after that there was a decrease in reaction until the 6th to 10th day. Additional injections caused no further diminution, each one inducing approximately

* Aided by a grant from the Venereal Disease Division of the United States Public Health Service.

[1] Heyman, A., *Ven. Dis. Inform.*, 1945, **26**, 51.

† This vaccine was prepared in the laboratories of the Georgia State Department of Public Health. It is ordinarily used for human immunization and fever therapy.

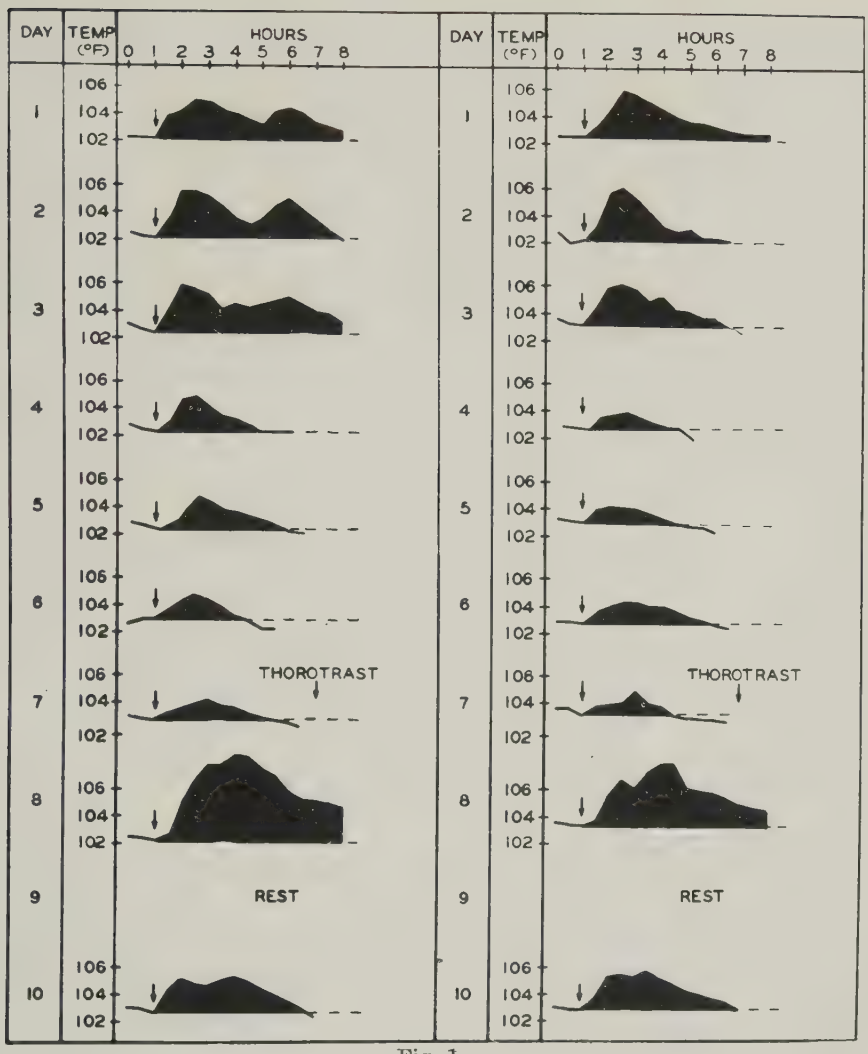

Fig. 1.

Temperatures of 2 rabbits given daily injections of same dose of typhoid vaccine. After development of tolerance, reticulo-endothelial blockade with Thorotrast caused a marked alteration in their temperature responses.

the same amount of fever, *i.e.*, a rise of 1.5-2.5°F, with return to the starting temperature in 3 to 5 hours. Four animals received daily injections for the full period of 45 days. After the usual decline in response during the first week these continued to respond to each dose of pyrogen with low grade fevers of about the same extent.

In other experiments longer intervals were allowed to elapse between injections of vaccine. Four rabbits which were injected once a week for 4 months developed some, but not a very considerable, tolerance. Another group of 4 rabbits received vaccine twice a week. A gradual decline in febrile response occurred during the first 8 to 10 injections, after which they reacted to each dose with about the same degree of fever, intermediate between an initial reaction and the minimal reaction noted in animals injected daily. At the end of 4 months the interval was shortened to one day, and they all showed a definite further diminution in their febrile responses.

Reticulo-endothelial blockade produced a

striking effect on the temperature responses of rabbits that had been previously "trained" by repeated daily injections of vaccine. A typical result is illustrated in Fig. 1. Here it will be noted that a considerable modification in response was manifest by the 7th day. Each animal was then given Thorotrast. On the following day administration of typhoid vaccine caused high, prolonged temperature elevations. After a rest of one day their febrile responses to the vaccine were again considerably lessened. This brief effect of the blocking agent is in line with other experience on functional interference with the reticulo-endothelial system.[2]

[2] Jaffe, R. H., *Physiol. Rev.*, 1931, **11**, 277.

These experiments show that rabbits can develop a tolerance to typhoid bacterial pyrogen, and that the tolerance is most marked when injections are given frequently. The mechanism of development of this state is not yet explained. Certain other observations, which cannot be given in detail here, indicate that the production of specific humoral antibodies is not responsible, and furthermore, that the development of tolerance to typhoid vaccine carries with it a similar alteration in response to other bacterial pyrogens. Possibly the process involves a change in the functional activity of the reticulo-endothelial system, providing for more rapid disposal of the foreign material.

15292

Effect of Penicillin on Blood Urea in the Rat

JACK RALPH LEONARDS AND FLORENCE WILLIAMS.
(Introduced by Victor C. Myers.)

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Early in 1943 the Floreys[1] reported that 4 out of 5 patients treated with 120,000 or more Oxford units of penicillin manifested a rise in blood urea. The elevated values returned rapidly to normal when the drug was withdrawn. Although no explanation was advanced for this phenomenon it was not thought to be due to renal damage.

The present study demonstrates that penicillin has no significant effect on the blood urea level of normal rats even though exceedingly large amounts of the drug are administered.

The penicillin employed was a rather impure preparation assaying 300 Oxford units per mg of total solids.* Normal adult albino rats of both sexes were used and the penicillin was injected intraperitoneally in a solution containing 25,000 Oxford units per ml. Samples of blood (0.1 or 0.2 ml) for urea determinations were obtained from the tail vein directly into a pipette and the urea was analyzed by the colorimetric method described by Ormsby.[2]

The results are presented in Table I. It is immediately evident that no significant increase in blood urea results even after the administration of 500,000 Oxford units per kg of body weight. This dosage represents the largest amount of our preparation of penicillin that could be tolerated by the rat without any apparent toxic manifestations. It should be emphasized that this is a tremendously large dose. When a group of rats were given 1,000,000 Oxford units of penicillin per kg of body weight over a period of 10 hours, 4 of the 6 rats died on the same day and the 2 surviving animals were severely

[1] Florey, M. E., and Florey, H. W., *Lancet*, 1943, **1**, 387.

* Kindly supplied by Ben Venue Laboratories, Inc., Bedford, Ohio.

[2] Ormsby, A. A., *J. Biol. Chem.*, 1942, **146**, 595.

TABLE I.
Average Blood Urea Levels Following Intraperitoneal Injections of Penicillin.

Penicillin Oxford units per kg Time after penicillin	No. of rats	Blood urea mg per 100 cc 0 hr	3 hr	7 hr	11 hr	30 hr
0	10	37	34	29	36	37
50,000*	6	34	32	29	28	30
100,000†	6	41	31	31	31	40
250,000†	6	38	51	—	52	38
500,000†	8	39	36	41	49	40

* Single injection.
† Divided into 5 equal doses. Two hours apart.

ill for several days.

Summary. The intraperitoneal injection of penicillin into adult albino rats had no significant effect on the blood urea level, even when doses as high as 500,000 Oxford units were administered per kg of body weight.

15293

Nervous System Mechanism for Epinephrine Secretion.

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The rate of liberation of epinephrine from the adrenal glands, under ordinary experimental conditions, has been determined as an average of about 0.00025 mg per kg of body weight per minute, in cats and dogs.[1] In the absence of experimentally induced alteration of the rate of secretion, the range of physiological epinephrine output was found to be approximately 0.0001 to 0.001 mg per kg per minute.

This spontaneous, constant secretion of epinephrine is sustained through the influence of a centre, or centres, located in the upper portion of the thoracic division of the spinal cord and can be considered as the normal, physiological secretion. Transection of the cord between the last cervical and the fourth dorsal segments abolishes epinephrine secretion from the adrenals. This centre is bilateral; semisection of the spinal cord, in this region, results in suppression of epinephrine secretion from the ipsilateral adrenal without detectable influence upon the secretion from the contralateral gland.[2]

Incidental observations made in the course of some other investigations suggested the probability of the existence of an inhibitory centre for epinephrine secretion, located in the brain. For example, the large increase in epinephrine output which is induced by the action of strychnine usually is preceded by a preliminary decline in the rate of secretion if a minimal effective dose of the drug is injected intravenously, or a larger dose

* Supported by the G. N. Stewart Memorial Fund, aided by grants from the Renziehausen and the Sarah Mellon Scaife Foundations.

A preliminary note was published in the *Proc. Am. Physiol. Soc.*, 1931. The study was interrupted when the Department of Experimental Medicine, at Western Reserve University, was discontinued. Some of the experiments were performed at the University of Chicago. The investigation was resumed and continued in this laboratory.

1 Stewart, G. N., and Rogoff, J. M., a. *J. Pharm. and Exp. Therap.*, 1916, **8**, 479; b. *Am. J. Physiol.*, 1923, **66**, 235.

2 Stewart, G. N., and Rogoff, J. M., a. *J. Exper. Med.*, 1917, **26**, 613; b. *Am. J. Physiol.*, 1920, **51**, 484.

subcutaneously. Intravenous administration of the larger doses generally causes only the augmentation of epinephrine output.[3] This suggests a possible activity of an inhibitory mechanism during the phase of cerebral stimulation by strychnine. Such an inhibitory influence upon epinephrine secretion would be submerged by the more powerful action of the opposing spinal cord centre, when the predominant influence of strychnine on the cord is effected.

Other observations likewise indicated the possibility that an inhibitory centre, which influences epinephrine secretion, exists in the brain. For example, when experimental animals were rendered insensitive to pain by compression or destruction of the brain, after preliminary anesthesia, the epinephrine output from the adrenal glands was found to be within but near or at the maximum of the range of spontaneous liberation.[2a,8] Furthermore, the same observation was made when the rate of liberation of epinephrine was determined in animals with cerebral anemia induced by occlusion of the blood supply to the brain.[4]

In the present series of experiments, the rate of epinephrine secretion from the adrenal glands was determined before and after transection at various levels of the brain. Decerebration was performed with the special knife described by Karrer and Stevens.[5] All of the experiments were performed on cats. The animals were anesthetized with urethane administered by stomach tube. The results obtained are illustrated in Table I and Fig. 3.

The level of decerebration was verified at the end of each experiment, by post-mortem examination. In Experiments 1-16, inclusive, transection of the brain was at levels bounded by the superior colliculus and the optic chiasm. In the others, decerebration was at various levels anterior to this. In 6 of the 28 animals, a small area of cortex escaped section (No. 7, 17, 18, 20, 24, 26).

The rate of secretion of epinephrine was assayed by the method of Stewart and Rogoff. Adrenal blood specimens were collected via the "cava pocket," before and after transection of the brain. The amount of blood obtained and the duration of the collection were measured, thus determining the rate of blood flow from the adrenals. The concentration of epinephrine in the blood specimens was assayed by the quantitative inhibition of rabbits' intestine segments. From this information, the rate of epinephrine secretion was readily calculated. In a few of the experiments, assay of the adrenal blood specimens was confirmed employing the "sensitized eye" method.[6]

Table I illustrates the decided increase in the rate of liberation of epinephrine which follows transection of the brain between the superior colliculus and the optic chiasm (cats 1-16, inclusive). In 12 of the animals the epinephrine output rose to or near the maximum of the normal range of spontaneous liberation. In cats 1 and 15, the output did not approach the upper limit of the range, although there was an increase of 2 and 3 times the initial rate of epinephrine secretion.

In one cat (8) there was, if anything, a slight reduction in the output. The adrenal blood flow, following decerebration, was very low in cats 4, 8 and 15. Brain transection was associated with excessive hemorrhage in these animals. However, experimental shock due to loss of systemic blood was not found to alter the epinephrine output from the adrenal glands.[7]

The 12 experiments in which transection of the brain was performed through or anterior to the optic chiasm showed no significant alteration of the rate of liberation of epinephrine from the adrenals, except in cat 24. In this animal, a reduction to about one-fourth of the initial epinephrine output was found, although the blood flow from the adrenals was good. A reduction in output to about one-half was found in cat 17 but

[3] Stewart, G. N., and Rogoff, J. M., *J. Pharm. and Exp. Therap.*, 1919, **13**, 95.

[4] Rogoff, J. M., *Am. J. Physiol.*, 1924, **67**, 551.

[5] Karrer, E., and Stevens, H. C., *J. Lab. Clin. Med.*, 1928, **14**, 266.

[6] Rogoff, J. M., PROC. SOC. EXP. BIOL. AND MED., 1937, **36**, 441.

[7] Stewart, G. N., and Rogoff, J. M., *Am. J. Physiol.*, 1919, **48**, 22.

TABLE I.
Epinephrine Output from the Adrenals after Decerebration.*

	Before brain section							After brain section				
				Epinephrine						Epinephrine		
No.	wt kg	Blood pressure mm Hg.	Blood flow g/min	Concentration x 100,000	Output μg/min	μg/kg/min	Interval min	Blood pressure mm Hg.	Blood flow g/min	Concentration x 100,000	Output μg/min	μg/kg/min
1	3.0		2.32	1:64	0.33	0.11	10		2.31	1:21	1.1	0.37
2	2.9	86	1.36	1:32	0.42	0.14	12	90	1.87	1:8.3	2.25	0.8
3	2.9	120	3.28	1:58	0.57	0.2	5	78	2.45	1:8.5	2.88	1.0
4	2.7	93	2.52	1:80	0.31	0.11	15	40	0.62	1:30	0.21	0.09
5	3.2	156	7.09	1:52	1.37	0.43	5	96	5.8	1:20	2.9	0.91
6	3.2	140	6.2	1:46	1.35	0.42	10	68	2.25	1:6.5	3.46	1.08
7	3.3	154	6.8	1:33	2.06	0.62	8	44	1.07	1:4.3	2.49	0.75
8	2.3	142	3.0	1:35	0.86	0.37	8	38	0.54	1:10	0.54	0.23
9	2.4	120	5.2	1:42.5	1.22	0.51	10	72	2.88	1:7.5	3.84	1.6
10	2.7	97	4.3	1:51	0.84	0.31	8	52	2.7	1:7.0	3.86	1.43
11	2.7	74	2.08	1:32.5	0.64	0.24	4	58	1.55	1:9.0	1.72	0.64
12	3.1	150	2.7	1:45	0.6	0.19	6	77	1.0	1:4.2	2.38	0.77
13	2.8	85	4.2	1:47.5	0.88	0.31	5	88	4.5	1:11	4.1	1.46
14	2.7	102	7.1	1:90	0.79	0.29	7	93	7.0	1:21	3.33	1.23
15	3.3	118	3.8	1:65	0.58	0.18	4	44	0.46	1:4.2	1.1	0.33
16	2.8	96	2.6	1:30	0.87	0.31	6	90	2.51	1:8.75	2.87	1.03
17	2.6	95	1.74	1:21	0.83	0.32	15	36	0.45	1:10	0.45	0.17
18	2.7	150	4.41	1:53	0.83	0.31	10	84	2.48	1:37.5	0.66	0.24
19	2.3	125	4.6	1:70	0.66	0.29	7	64	2.0	1:31	0.64	0.28
20	2.2	90	3.1	1:30	1.03	0.47	8	68	1.3	1:9.7	1.34	0.61
21	2.9	145	5.9	1:55	1.08	0.37	5	126	4.4	1:35	1.26	0.43
22	3.2	122	6.41	1:56	1.14	0.36	5	84	2.93	1:22.5	1.3	0.41
23	2.4	130	6.2	1:55	1.13	0.47	4	95	3.82	1:33	1.16	0.48
24	3.7	112	4.4	1:37	1.19	0.32	5	88	2.4	1:70	0.34	0.09
25	3.1	130	2.16	1:25	0.86	0.28	5	66	1.01	1:12.8	0.79	0.25
26	2.9	122	3.8	1:40	0.95	0.33	4	70	1.15	1:9.75	1.18	0.41
27	3.9	110	3.2	1:28	1.14	0.29	6	82	2.9	1:20	1.45	0.37
28	2.5	122	3.41	1:52	0.66	0.26	6	97	2.86	1:40	0.71	0.28

* In cats 1-16, inclusive, decerebration was between the superior colliculi and the optic chiasm; in all the others, it was anterior to that level.

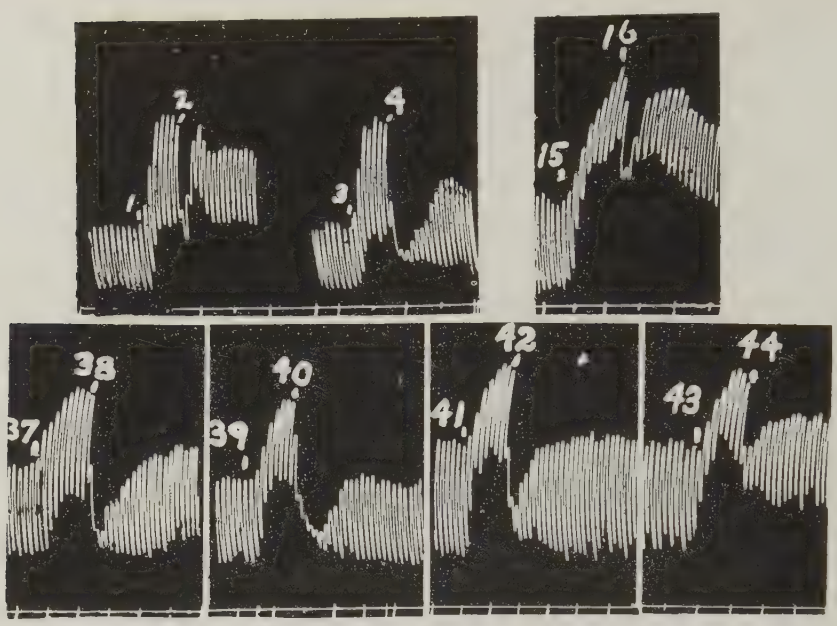

Fig. 1.

At the uneven numbers Ringer's solution was displaced by indifferent blood and this at 2 by adrenal blood specimen A, at 4, 38 and 42 by adrenal blood specimen B, at 16 by indifferent blood to which was added adrenalin to make a concentration of 1:7,000,000, at 40 a concentration of 1:1,450,000, and at 44 a concentration of 1:2,850,000. A was assayed at 1:6,400,000, and B at 1:2,100,000. Time in half-minutes. Reduced to two-thirds.

in this case, as in cats 4, 8 and 15, the adrenal blood flow was very slow. The question may be raised, in these cases, whether trauma of the brain, in a region near the inhibitory centre for epinephrine secretion, might not result in stimulation of that centre and consequent reduction of epinephrine output from the adrenal glands.

In 2 experiments (cats 3 and 5), in which the decerebration was between the superior colliculus and the optic chiasm, and in one (cat 19), in which the decerebration was anterior to this region, additional adrenal blood specimens were obtained for assay. In cat 3, a specimen obtained 25 minutes after decerebration, when the adrenal blood flow was 1.15 g per minute, was assayed at 1:450,000 adrenalin, corresponding to an output of 0.0025 mg per minute for the cat or 0.00086 mg per kg per minute. In cat 5, a specimen obtained 18 minutes after decerebration, when the adrenal blood flow was 1.02 g per minute, was assayed at 1:750,000 adrenalin, corresponding to an output of 0.00136 mg per minute for the cat or 0.00048 mg per kg per minute. In the case of transection of the brain through the optic chiasm (cat 19), a specimen obtained 35 minutes after decerebration, when the adrenal blood flow was 1.4 g per minute showed the same epinephrine output from the adrenals as before and 7 minutes after the decerebration.

To illustrate the results obtained, condensed protocols and some of the tracings (Fig. 1, 2) from the assay in 2 of the experiments are given (cats 1 and 13). In these animals the transection of the brain was between the superior colliculi and the optic chiasm. Cat 1 was a pregnant female and cat 13 a male. These animals were selected for illustration because the results are unequivocal. The rate of blood flow from the adrenal glands did not change after decerebration; nevertheless, the epinephrine concentration in the adrenal blood specimens obtained after the decerebration (B) was decidedly greater than that in the initial specimens (A).

Condensed protocol. Cat 1: pregnant fe-

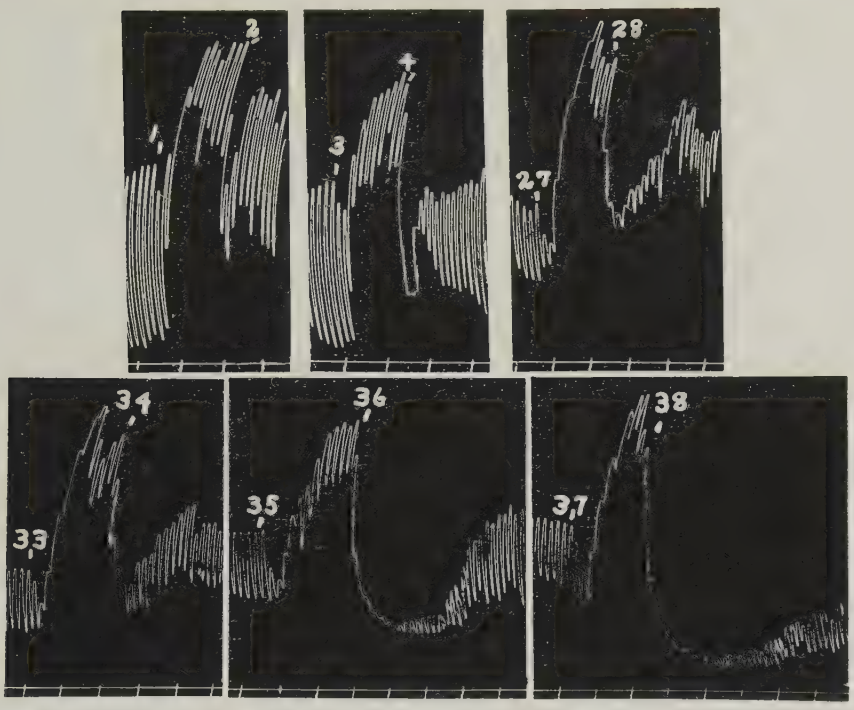

Fig. 2.

At the uneven numbers Ringer's solution was displaced by indifferent blood and this at 2 and 34 by adrenal blood specimen A, at 4 and 36 by adrenal blood specimen B, at 28 by indifferent blood to which was added adrenalin to make a concentration of 1:6,250,000, and at 38 a concentration of 1:700,000. A was assayed at 1:4,750,000, and B at 1:1,100,000. Time in half-minutes. Reduced to three-fifths.

male; weight 3.0 kg.

a.m.

8:45; Urethane (20 cc of 20% sol.) by stomach tube.

10:45; Anesthesia complete.

11:22; Collected adrenal blood specimen A, 5.8 g in 2½ min.; blood flow, 2.32 g per minute.

11:40; Inserted tracheal cannula and trephined skull.

11:42; Decerebration.

11:52; Collected adrenal blood specimen B, 5.78 g in 2½ min.; blood flow, 2.31 g per minute.

Complete transection of the brain, just anterior to the superior colliculi, verified by post mortem examination.

Specimen A, assayed at 1:6,400,000 adrenalin, corresponding to an output of 0.00033 mg per minute for the cat or 0.00011 mg per kg body weight per minute. Specimen B, assayed at 1:2,100,000 adrenalin, corresponding to an output of 0.0011 mg per minute for the cat or 0.00037 mg per kg body weight per minute (Fig. 1).

Condensed protocol. Cat 13; male; weight, 2.8 kg.

a.m.

9:30; Urethane (25 cc of 20% sol.) by stomach tube.

10:30; Anesthesia complete.

10:55; Collected adrenal blood specimen A, 8.4 g in 2 min.; blood flow, 4.2 g per minute.

11:12; Inserted tracheal cannula and trephined skull.

11:18; Decerebration.

11:23; Collected adrenal blood specimen B, 9.0 g in 2 min.; blood flow, 4.5 g per minute.

Complete transection of the brain, about ⅛ inch anterior to the superior colliculi, verified

TABLE II.
Statistical Summary of Epinephrine Output.

Cats	Decerebration	Mean μg/kg/min	Squared standard deviation	Probable error of mean	Ratio of std.error of diff. to diff. of means		t	
					Observed	Significant	Observed	Significant
1-16	before	0.296	0.146	0.025				
1-16	after	0.858	0.451	0.076	7.02	3.0	4.736	2.131
17-28	before	0.339	0.069	0.013				
17-28	after	0.335	0.144	0.028	0.031	3.0	0.891	2.201
1-16	before	0.296	0.146	0.025				
17-28	before	0.858	0.069	0.013	1.529	3.0	0.939	2.201

by post mortem examination.

Specimen A, assayed at 1:4,750,000 adrenalin, corresponding to an output of 0.00088 mg per minute for the cat or 0.00031 mg per kg body weight per minute. Specimen B, assayed at 1:1,100,000 adrenalin, corresponding to an output of 0.0041 mg per minute for the cat or 0.00146 mg per kg body weight per minute (Fig. 2).

Although the results are sufficiently definite to support our conclusions, they are presented in the following statistical summary (Table II) for which we are greatly indebted to Dr. Paul L. McLain of the Department of

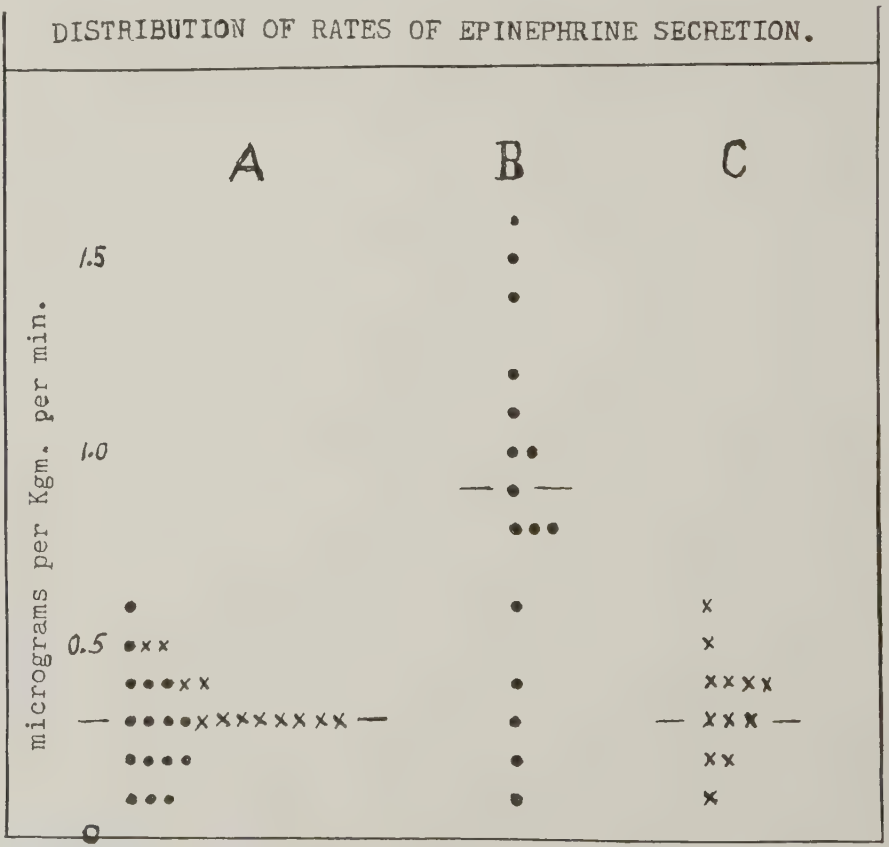

Fig. 3.

A—Before decerebration.
B—After decerebration between superior colliculi and optic chiasm.
C—After decerebration anterior to level of B.
●—Cats 1-16, inclusive.
X—Cats 17-28, inclusive.

Physiology. The distribution of the rates of epinephrine output is illustrated in Fig. 3.

Summary. These experiments indicate the existence of an inhibitory central mechanism, located in the region of the brain bounded by the superior colliculi and the optic chiasm, which influences epinephrine secretion from the adrenal glands. The existence of a central mechanism in the spinal cord, which exercises an opposite influence to that of this cerebral centre, was established earlier.[2] Removal of the influence of the brain centre, results in epinephrine output from the adrenals at the maximum rate of spontaneous liberation which can be sustained through the physiological activity of the spinal cord centre. This rate corresponds with the upper limit of the range of normal epinephrine secretion.[1]

Despite the fact that epinephrine was the first hormone to be isolated from its source, and to be synthesized, the functional significance of epinephrine secretion is still obscure. Physiological interpretations from pharmacological observations often have been misleading. Only quantitative experiments, yielding results within the limits of the physiological rate of liberation, can be expected to yield unequivocal information on the function of epinephrine secretion.

In the light of the experiments reported herewith, it can be seen why reflex augmentation of the rate of epinephrine secretion has not been demonstrated successfully in animals under the influence of asphyxia[8] or stimulation of sensory nerve trunks.[9] For, even if these stimuli might affect appropriate receptors, which could alter the rate of epinephrine secretion, there is no reason to assume that they might not equally influence the cerebral and the cord centres, thus defeating the possibility of eliciting reflexly either augmentation or diminution in the rate of epinephrine secretion from the adrenal glands. In preliminary tests, it was not found possible to elicit augmentation of the epinephrine output by stimulation of sensory nerves, following mechanical destruction or compression of the brain.[9a]

[8] Stewart, G. N., and Rogoff, J. M., *J. Pharm. and Exp. Therap.*, 1917, **10**, 49.

[9] Stewart, G. N., and Rogoff, J. M., a. *J. Exper. Med.*, 1917, **26**, 637; b. *Am. J. Physiol.*, 1924, **69**, 605.

15294 P

Inhibition of Experimental Drug Allergy by Prior Feeding of the Sensitizing Agent.

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In the course of studies on experimental drug allergy, it was noticed that sensitization was apt to be less successful in guinea pigs which previously had been treated briefly with the same chemical. Experiments were undertaken to establish at will such a refractory state.* For the preparatory, or blocking, treatment, the methods tried included the parenteral injection of soluble and insoluble protein conjugates of the incitant itself, repeated cutaneous application of the chemicals in very low (non-sensitizing) concentration, intravenous injections, and feeding experiments. After this preliminary treatment, a rest period of 2 weeks was allowed, and then the animals, in parallel with fresh guinea pigs as controls, were subjected to an active sensitizing course consisting of 6 or more intracutaneous injections of the incitant over a period of several weeks. Following

* In the case of neoarsphenamine, Sulzberger[4] had found that a single intravenous injection of the substance usually prevented the active sensitization sought by an intracutaneous injection made one day previously.

TABLE I.

Hypersensitivity rated as:	Prior feeding of allergen (93 animals)	Controls (77 animals)
	%	%
High	3.2	74.0
Good	0.0	16.9
Moderate	8.6	5.2
Weak	20.4	3.9
Low	46.2	0.0
Very faint, or entirely negative	21.5	0.0

another 2 weeks' rest, the outcome of the challenging sensitization was determined by a "contact test" in which the skin was painted with dilutions of the incitant in olive oil and the animals were examined for the development of cutaneous reactions.

While several substances were employed in the first experiments, the compound 2:4 dinitrochlorobenzene was chosen for chief attention because it is actively allergenic and is well known in the experimental sensitization of human beings.[1,2,3] Rather unexpectedly, and in sharp (but not absolute) contrast to all other methods employed, a blocking effect of substantial degree was found to be induced by feeding the chemical. A 1% solution of dinitrochlorobenzene in olive oil was fed from the tip of a pipette in such manner that contact of the solution with the muzzle was minimal. The feedings (0.3 cc) were made daily for 6 days, followed by an 8-day rest period; 2 or 3 such courses were given prior to the attempt at sensitization by intracutaneous injections. While the latter regularly sensitized either normal animals or animals fed the vehicle alone (olive oil), a very considerable diminution in effect was encountered in groups that had received prior feedings of the chemical. This is shown in Table I, which brings together the results from various, essentially similar experiments. It will be seen that the protection afforded was not absolute, although in some experiments well-nigh complete inhibition was attained. On the other hand, it remains possible that the protective effect may be overridden by intensive treatment with the allergen.

To examine whether the feedings had led to a blocking mechanism directed only towards the same chemical or had induced a non-specific resistance to sensitizations, the behavior of the animals to an active sensitizing course with a second, unrelated substance (*o*-chlorobenzoyl chloride) was examined. The resistant and "positive control" groups did not differ at all in their response to the new agent. This held true, also, when animals fed with one chemical then received a simultaneous series of intracutaneous injections with 2 substances, one, the specific allergen, and the other, a non-related compound. The inhibitory effect therefore was specific, and the animals were normal in their response to another compound.

That the effect is rather durable, and one apparently not dependent upon retention of the ingested substance, was demonstrated in one lot of 30 animals fed 2:4 dinitrochlorobenzene. At varying intervals, groups of approximately 10 animals were given the sensitizing course, along with an equal number of new animals as controls. Protection was still apparent even in the last group tested: here the interval between the final feeding and the beginning of the challenging course had been 27 weeks, and the final contact test was made 4 weeks later.

It is possible, then, to induce a profound and lasting protection against experimental sensitization when one starts with animals which are not already sensitive to the substance in question; this is consonant with the experiment of Sulzberger.[4] On the other hand, courses of feeding given to animals previously made sensitive to 2:4 dinitrochlorobenzene have so far shown no appreciable effect in diminishing the degree of hypersensitivity, a result in accord with attempts to decrease an established sensitivity in guinea pigs by parenteral injection of soluble protein

[1] Wedroff, N. S., and Dolgoff, A. P., *Arch. Derm. u. Syph.*, 1935, **171**, 647.

[2] Landsteiner, K., Rostenberg, A., and Sulzberger, M. B., *J. Invest. Dermat.*, 1939, **2**, 25.

[3] Haxthausen, H., *Acta Dermato-Venercol.*, 1939, **20**, 257.

[4] Sulzberger, M. B., *Arch. Derm. and Syph.*, 1930, **22**, 839.

conjugates of the incitant.[5] There are, however, statements in the literature to the effect that sensitive human beings have been partially desensitized by ingestion of the specific allergen.[6,7]

Summary. Through the feeding of certain allergenic compounds to the non-sensitive subject, a state of resistance may be established against subsequent experimental sensitization of the skin by the same substance.

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[7] Stevens, F. A., *J. Am. Med. Assn.*, 1945, **127**, 912.

15295

Rabbit Papilloma and Vaccinia Viruses and T_2 Bacteriophage of *E. coli* in "Shadow" Electron Micrographs.*

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Although electron micrography has added greatly to knowledge of the shape, size and other characters of viruses, the findings leave much to be desired. The capacity of viruses to absorb and scatter fast electrons is relatively small, and, consequently, images of the particles are likely to be of low contrast and indistinct, especially at the periphery. Contrast in influenza virus images may be enhanced slightly by treatment of the virus with osmic acid;[1] use of calcium chloride, though obscuring internal structure, aids greatly in micrography of equine encephalomyelitis[2] and influenza viruses[3] but not in the instances of the rabbit papilloma and tobacco mosaic viruses. In addition, the images are flat, and judgment of particle shape must be based solely on contour portrayed in 2 dimensions. A considerable advance in the electron micrography of viruses is the application of the shadow-casting technic recently reported by Williams and Wyckoff.[4,5,6] In this process, the molecules of metals evaporated *in vacuo* fall on the virus particles at a grazing angle and cast "shadows" which give the appearance of 3-dimensional contour to images in electron micrographs. The shape and length of the shadows cast on the background and the contour of the images afford a much better basis for judgment of the shape of the particle than the unshadowed images. Studies with this technic have been made of the vaccinia and rabbit papilloma viruses and the T_2 bacteriophage of *E. coli*. The results‡ with these 3 viruses are reported in the present paper.

Materials and Methods. The viruses were

* This work was aided by the Commission on Influenza and the Commission on Epidemiological Survey, Board for the Investigation and Control of Influenza and Other Epidemic Diseases in the Army, Preventive Medicine Service, Office of the Surgeon General, United States Army, and by a grant to Duke University from Lederle Laboratories, Pearl River, New York.

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[1] Sharp, D. G., Taylor, A. R., McLean, I. W., Jr., Beard, D., Beard, J. W., Feller, A. E., and Dingle, J. H., *J. Immunol.*, 1944, **48**, 129.

[2] Sharp, D. G., Taylor, A. R., Beard, D., and Beard, J. W., *Arch. Path.*, 1943, **36**, 167.

[3] Taylor, A. R., Sharp, D. G., Beard, D., Beard, J. W., Dingle, J. H., and Feller, A. E., *J. Immunol.*, 1943, **47**, 261.

[4] Williams, R. C., and Wyckoff, R. W. G., PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 265.

[5] Williams, R. C., and Wyckoff, R. W. G., *Nature*, 1945, **156**, 68.

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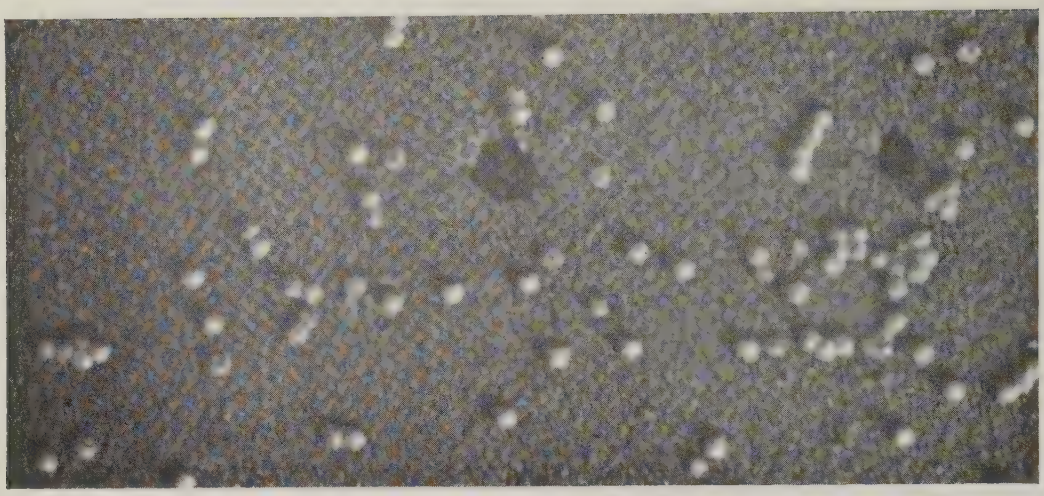

Fig. 1.
Rabbit papilloma virus shadowed with 10 mg of gold at the angle tangent 2/7. Magnification 42,700 x.

employed only in purified preparations. Vaccinia[7,8] and rabbit papilloma viruses[9,10] were obtained as previously described. The bacteriophage,§ the T_2 strain[11] parasitizing *E. coli,* was cultivated on the host grown in broth and purified by ultracentrifugal procedures which will be described elsewhere.[12] Purified virus suspensions containing about 10^{12} particles per ml were placed on the collodion film support, and, after about half a minute, the excess fluid was removed with a fine pipette. The films after drying were viewed immediately in the electron microscope or, more often, lightly washed by flooding once or twice with or dipping in distilled water. When the films were again dry, they were ready for observation and electron micrography in the ordinary way, or for application of evaporated metal. In some instances the films were observed and photographed, then shadowed and examined again.

Gold was used for the papilloma and vaccinia viruses and chromium for the bacteriophage. The weighed metals were evaporated in a 10-turn conical helix of 5 mil tungsten wire, through which alternating current was passed from a 500-volt-ampere transformer at 20 volts. Enough heat was generated to complete the evaporation in 1 to 3 minutes. The vacuum chamber was a glass tube 1.5 inches in diameter and 12 inches long. It was continuously evacuated with a Cenco Hivac pump and a 2-stage mercury diffusion pump, giving an ultimate vacuum of the order of 10^{-5} mm of Hg. The metal was deposited on the papilloma and vaccinia viruses at the angle whose tangent was 2/7 and on the bacteriophage at the angle of tangent 1/5. The distance from the evaporator coil to the virus was 7 cm. Assuming evaporation equally in all directions, the metal thickness on the side of the virus particle normal to the beam would be given by the equation

$$\text{Thickness} = \frac{W}{4\pi r^2 \rho}$$

Science, 1945, **101**, 596.

‡ This work was described in part at the meeting of the Electron Microscope Society of America, December 1, 1945, at Princeton, N. J.

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8 Beard, J. W., Finkelstein, H., and Wyckoff, R. W. G., *J. Immunol.,* 1938, **35**, 415.

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§ We are greatly indebted to Dr. Max Delbrück, Vanderbilt University, for the strain of bacteriophage and its host and for advice in the problems of cultivation.

11 Anderson, T. F., *J. Cell. and Comp. Physiol.,* 1945, **25**, 17.

12 Hook, A. E., Taylor, A. R., Sharp, D. G., and Beard, J. W., to be published.

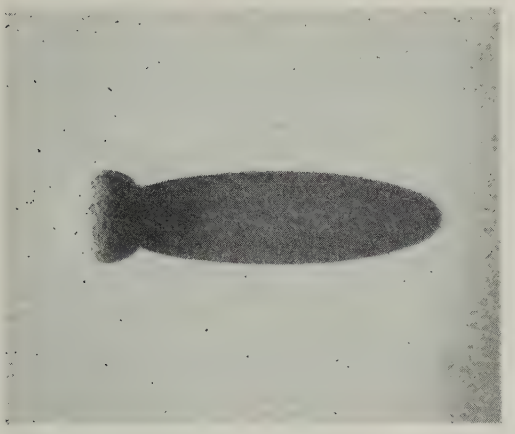

Fig. 2.
Photograph of sphere (tennis ball) and the shadow cast by light striking at the angle tangent 2/7.

in which W = weight of metal evaporated in grams; r = distance (7 cm) from the metal to the virus preparation and ρ = the density of the metal (19 for gold, 7 for chromium). The thickness of the deposit on the film lying at an angle φ to the beam was less by the factor tan φ, which in this case was 2/7 or 1/5. Under the conditions of the experiments the thickness of the layer of gold on the normal surface was about 8.5 Å per mg of metal evaporated; the values for chromium were greater by the quotient of the densities, or 2.7 times as much.

For purposes of comparison, a photograph was made of a spherical object, a tennis ball, and the shadow produced by light striking it at the angle of tan 2/7.

Results. The rabbit papilloma virus in conventional electron micrographs[13] is seen as circular or rounded images of low contrast which merge indistinctly into the background at the periphery. On the basis of apparent uniformity in the size and shape of the images, the virus was judged to be essentially spherical in shape. In Fig. 1 there is shown the appearance of the virus shadowed with 10 mg of gold. Most of the images are highly uniform in shape and size; others, which are irregular in size and shape, have the appearance of representing fragments of virus particles.

The characters of the shadow cast by a sphere, a tennis ball, lighted at an angle of tangent 2/7, are shown in Fig. 2. On comparison of Fig. 1 with Fig. 2, it is seen that the shadows of the virus particles differ greatly from the shadow cast by the ball. The shadows of the virus are nearly triangular in shape, relatively very broad at the base, and the length is, on the average, about ⅔ of what it would be if the height of the particles were equal to the diameter of the images. Judging from these characters, it is clear that the particles, at the moment of shadowing, were not spherical, but were flattened on the under and possibly on the upper sides as well.

Vaccinia virus deposited on the collodion membrane from 0.005 M phosphate buffer and washed by dipping in water is pictured in Fig. 3. As described by Green, Anderson and Smadel,[14] the images are approximately rectangular in shape and give evidence of differentiation in the internal structure of the particle as shown by the presence of rounded regions of density greater than that of the remainder of the particle. The particles lightly washed seem to be coated with a sticky material which causes coherence of some of the particles and in which bubble-like structures appear.

Vaccinial elementary bodies shadowed with 8 mg of gold are seen in Fig. 4. In contour in the horizontal plane the images of Fig. 4 are similar to those of Fig. 3 except for roughness and unevenness about the edges, which may be related either to uneven deposit of gold or possibly to coating of the bubble-like structures seen in Fig. 3. The bodies are clearly flattened in varying degree, as shown both by contour and length of the shadows. The shadows are not sharp in the region of the apex where the metal shades far into the shadow. One of the images seen in Fig. 4 is almost circular and of very high contrast. The shadow associated with it is much longer than the shadows of the other particles. This appearance suggests, as discussed below, that the image represents

[13] Sharp, D. G., Taylor, A. R., Beard, D., and Beard, J. W., PROC. SOC. EXP. BIOL. AND MED., 1942, **50**, 205.

[14] Green, R. H., Anderson, T. F., and Smadel, J. E., *J. Exp. Med.*, 1942, **75**, 651.

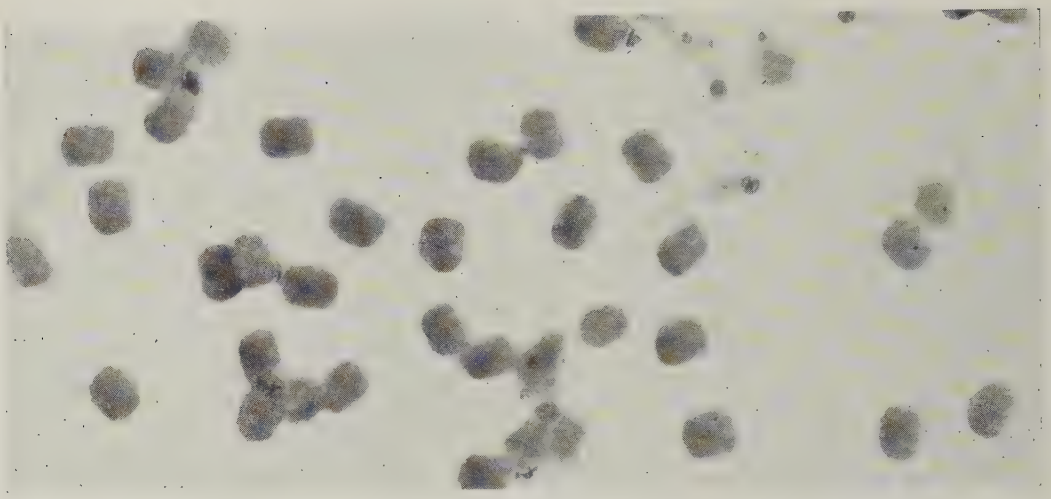

Fig. 3.
Conventional electron micrograph of vaccinial elementary bodies. Magnification 25,000 x.

one of the particles standing on end. In many of the images there are seen relatively broad and low mound-like protrusions that may represent the effects of internal structure, possibly the material of the regions of high density of Fig. 3, extending up beneath the surface.

The T_2 bacteriophage of *E. coli* is shown in the electron micrograph of Fig. 5. The purified bacteriophage was suspended in physiological saline solution, and the concentrate was diluted ⅕ with 0.023 M $CaCl_2$ for preparation of the film. Similar to the findings of Luria and Anderson,[15] the images of the bacteriophage are tadpole-shaped, showing the presence of a large head and a stubby tail tipped with a ball- or disc-shaped knob. The heads, which are of high capacity to absorb electrons, are uniform in size and hexagonal in shape. The tails, which show low electron-stopping power, are uniform in length but appear to vary in width. No internal structure was visible in the presence of the calcium salt.

In Fig. 6 are shown bacteriophage particles shadowed by the evaporation of 3.7 mg of chromium. The heads do not give the shadows either of ideal spheres, or even of regular polyhedrons, but rather those of short, somewhat flattened rods with conical caps. The stubby tail, of uneven thickness, terminates in a ball- or disc-shaped structure. The tail shadows are likewise short, indicating a flattened condition consistent with the observed low electron-stopping power. The degree of flattening is not the same in all preparations nor has it been the same for the individuals of a given picture, as may be seen in Fig. 6.

Discussion. The application of the shadow technic adds greatly to the results of direct observation of viruses in the electron microscope. Coating of the virus preparation is effected *in vacuo* under conditions not greatly different from those to which the preparation is subjected in the course of conventional electron micrography. Thus the characters portrayed with 3-dimensional effect in the shadow pictures are those of the dried and possibly shrunken and distorted virus particles and are probably not quantitatively representative of the particles in their native state in aqueous suspension. Measurements of shadows[6] of tobacco mosaic virus rods yield values of rod height similar to results obtained for rod width by means of X-ray examination[16] and measurements in electron micro-

[15] Luria, S. E., and Anderson, T. F., *Proc. Nat. Acad. Sci.*, 1942, **28**, 127.

[16] Bernal, J. D., and Fankuchen, I., *J. Gen. Physiol.*, 1941, **25**, 111.

[17] Stanley, W. M., and Anderson, T. F., *J. Biol. Chem.*, 1941, **139**, 325.

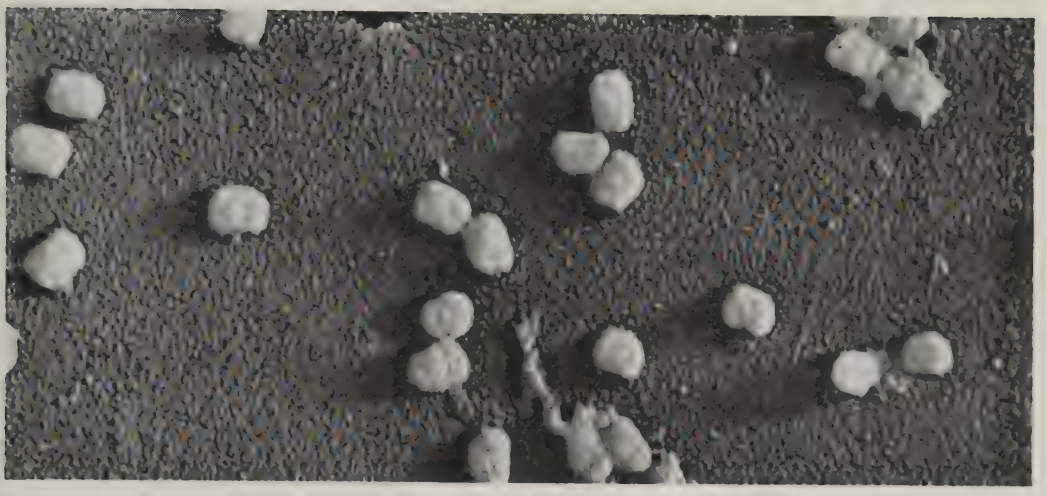

Fig. 4.
Vaccinia virus shadowed with 8 mg of gold at the angle tangent 2/7. Magnification 25,000 x.

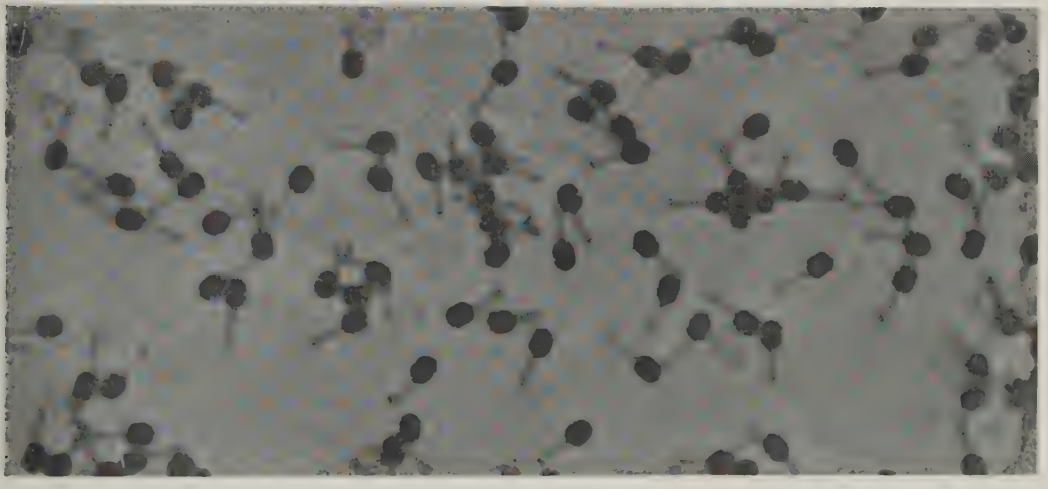

Fig. 5.
Conventional electron micrograph of the T_2 bacteriophage of *E. coli*. Magnification 42,700 x.

graphs.[17] That shrinkage of other viruses occurs under these conditions, however, is indicated by the observation of particle diameter in electron micrographs smaller than values obtained in studies of the virus in aqueous suspension.[18]

Shadow electron micrographs of the papilloma virus corroborate evidence of uniformity of particle size and shape obtained in sedimentation studies[19] and from conventional electron micrographs.[13] In the shadow micrography, the apparent height of the particles is about ⅔ that expected of spheres, a value much greater than that for oblate spheroids of axial ratio 11-1 previously predicted[19] from sedimentation, diffusion and viscosity data on the assumption that the particle is unhydrated. Recent studies[20] of

[18] Sharp, D. G., Taylor, A. R., McLean, I. W., Jr., Beard, D., and Beard, J. W., *J. Biol. Chem.*, 1945, **159**, 29.

[19] Neurath, H., Cooper, G. R., Sharp, D. G., Taylor, A. R., Beard, D., and Beard, J. W., *J. Biol. Chem.*, 1941, **140**, 293.

[20] Sharp, D. G., Taylor, A. R., and Beard, J. W., *J. Biol. Chem.*, 1946, **163**, 289.

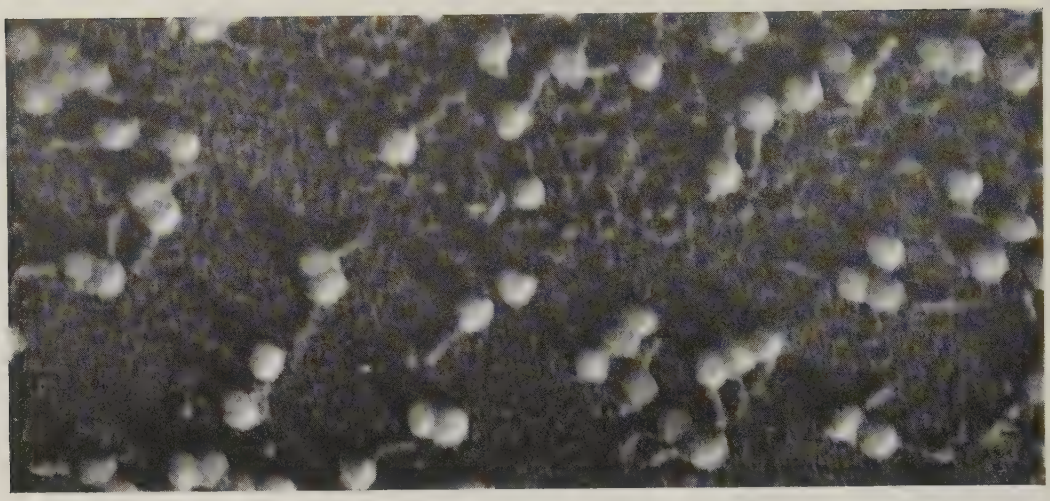

Fig. 6.
Bacteriophage shadowed with 3.7 mg of chromium at the angle tangent 1/5. Magnification 42,700 x.

the density of the papilloma virus in aqueous suspension indicate that the particles are 58% water (by volume) and, if spherical in shape, about 66 mμ in diameter. The difference between this value and 44 mμ observed in electron micrographs[13] is probably, in part, a measure of the shrinkage occurring in the plane of the picture. Greater shortening of the vertical diameter than of that in the horizontal plane is not improbable, since settling and spreading of the wet particle on the collodion membrane might be expected. This is consistent with the obvious flattening of the particles on the under side indicated by the shadows broad at the base. Quantitatively, the shadow pictures show that the dry papilloma virus particles are slightly flattened spheroids; the finding is not incompatible with the view that the virus is essentially spherical in the wet state and flattens on drying in contact with the collodion membrane.

The elementary bodies of vaccinia have been described as "brick-shaped"[14] which implies a difference between the height and the width of the particles lying on the collodion membrane. Judging from the contour in the shadow pictures, the dry bodies seem greatly flattened, but this appearance is not wholly consistent with the length of the shadows. This discrepancy and the lack of sharpness of the shadows may be explained, at least in part, on the assumption that shrinkage of the particle continues during the process of metal-coating. The particles, even after drying, are clearly well-rounded about all diameters. There is some evidence that the particles are not brick-shaped but are essentially short cylinders or rods. In a large number of micrographs, the width or short dimension of particles has been relatively uniform, and no evidence has been seen of bodies lying on edge. On the contrary, circular images of high contrast and of diameters similar to the short dimension of the rectangular images occur with a frequency of about 1 or 2 per hundred. A shadow image of this sort is shown in Fig. 4. The circular shape, and the relatively high contrast of the image, together with the greater length of the shadow, may be interpreted as evidence that this particle is a short rod or cylinder standing on end. The consistency of the findings suggests that the body casting this image differs from the others only in orientation, and that the vaccinia virus may be essentially rod-shaped.

The mound-shaped protrusions seen in the shadow images are interpreted as representing the intraparticular material of high contrast in conventional electron micrographs. The structure appears relatively large and of greater density and resistance to shrinkage than the remainder of the particle.

The highly uniform width, the well-rounded contours and the length of the shadows are characters suggesting that the headpieces of the bacteriophage are likewise probably short cylinders. The hexagonal shape in ordinary electron micrographs indicates a conical shape of the ends of the rods. In the absence of information of the density, size and water content of the bacteriophage in aqueous suspension, no estimate can be made of the possible degree of shrinkage on drying.

Summary. Electron micrographs of the rabbit papilloma and vaccinia viruses and the T_2 bacteriophage of *E. coli* were obtained with the metal-shadowing technic. The papilloma virus dried *in vacuo* for application of the metal appeared spheroidal in shape, flattened especially at the region of contact with the film. The vaccinia virus was greatly flattened and showed the presence of a denser internal material bulging beneath the surface. The bacteriophage was a tadpole-shaped structure with a headpiece of well-rounded contours and a stubby tailpiece. The findings were discussed with respect to their bearing on the shape of the viruses in the wet state.

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Attempts to Propagate Murine Poliomyelitis Virus on Various Intestinal Bacteria and Protozoa.*

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Studies of microbial metabolism have amply demonstrated that many bacteria and protozoa are endowed with a complex equipment of enzymes that are utilized for cellular propagation. It is therefore not surprising that numerous attempts have been made to grow certain pathogenic viruses in symbiosis with bacteria or other free-living cells.[1] Without passing on the merits of these observations, the relationship between the virus of poliomyelitis and the intestinal flora seemed to us of particular interest in this connection. As is well known, human virus—with sufficient neurotropic virulence to permit intracerebral transfer to monkeys—can often be isolated from the faeces of cases or healthy contacts. More important yet, normal albino mice harbor regularly in the intestinal tract neurotropic murine virus (Theiler virus) which apparently persists for the lifetime of the animal. Additional evidence of the remarkable affinity of poliomyelitis virus for the alimentary canal is furnished by experience with the mouse-adapted strain of human SK poliomyelitis virus which could be recovered from the faeces of 3 different infected hosts, natural or experimental, *i.e.*, man,[2] monkey[3] and guinea pig.[4] How a supposedly strictly neurotropic virus can maintain itself for any protracted period in the absence of living nervous material is dif-

* Aided by grants from the Philip Hanson Hiss, Jr., Memorial Fund, the Warner Institute for Therapeutic Research, and anonymous donors.

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2 Trask, J. D., Vignec, A. J., and Paul, J. R., PROC. SOC. EXP. BIOL. AND MED., 1938, **88**, 147; *J. Am. Med. Assn.*, 1938, **111**. 6.

3 Trask, J. D., and Paul, J. R., *Ann. Int. Med.*, 1942, **17**, 975.

4 Ibanez, J. S., Trabajos del Instituto Cajal de Investigaciones Biologicas, 1944, **36**, 137.

ficult to understand. The theory could therefore be advanced that the infectious agent may possess a vicarious enterotropism which permits viral propagation, at a saprophytic level, on enteric microorganisms normally present in the intestinal canal.

To test the validity of this hypothesis experiments were undertaken in which cultures of certain intestinal bacteria or protozoa (including some non-intestinal parasites)† were examined for their ability to serve as a substrate for *in vitro* propagation of murine poliomyelitis virus. A mouse-adapted strain of human poliomyelitis virus (MM) which grows well in embryonic mouse and fairly well in chick tissue cell culture was used in this work.[5] This virus was chosen because its extremely high virulence for mice by both intracerebral and intraperitoneal routes facilitated the detection of minimal amounts of virus; moreover, its marked resistance against physical and chemical agencies offered some assurance for survival of the virus under unfavorable environmental conditions. The microorganisms employed as test substrates for possible viral propagation were the following: (1) Mixed bacterial flora obtained from human faeces, (2) *B. coli communis,* (3) *Leptospira biflexa,* (4) *Entamoeba coli,* (5) *Trichomonas hominis,* (6) *Chilomastix mesneli,* (7) *Tetrahymena geleii,* (8) *Leishmania donovani,* (9) *Trypanosoma gambiense.* The general experimental procedure consisted of combining sufficient 10% viral mouse brain suspension with the different bacterial or protozoal cultures in their respective optimal media so as to yield a final 1:100 virus concentration. After proper incubation such mixtures were transferred to new culture media in amounts constituting a further 10-fold dilution of virus with serial passages being maintained for several subsequent generations. For control purposes, 10% viral brain suspension, in identical proportions, was added to the respective sterile culture media which, after similar incubation, were transferred serially to new culture media, thus yielding progressively increasing 10-fold dilutions of virus alone. The 2 sets of tubes, *i.e.,* microbial cultures and controls, were examined at each passage for the presence of viable organisms by microscopic count and for the presence of virus by the injection of albino mice. In the case of the enteric bacteria it was necessary to use Seitz filtrates for injection, whereas the protozoal culture and control tubes could be injected safely without previous filtration. All injections were carried out intracerebrally with 0.03 cc of test fluid, except for cultures of *Entamoeba coli, Chilomastix mesnili* and *Trichomonas hominis* which on account of their concomitant bacterial flora were tested by intraperitoneal injection of 0.1 cc amounts. The injected mice were carefully observed for the development of characteristic symptoms. Tissues of animals which died without definite signs of paralysis were further checked by subpassage to new mice for final identification of the virus. The results are shown in Table I.

It will be seen from Table I that none of the tested bacteria or protozoa of increasingly differentiated structure were capable of supporting neurotropic MM virus beyond the number of passages which maintained virus in control tubes, with the possible exception of the *Trichomonas hominis* culture which was therefore studied in more detail. Thus, active virus was clearly demonstrable in the protozoal-bacterial culture up to and including the fourth serial passage, whereas virus alone in the uninoculated medium failed to survive beyond the initial passage in repeated tests. The impression was first gained that virus had actually multiplied to some ex-

† Grateful acknowledgement is made of the receipt of the protozoal cultures from the following sources: *Entamoeba coli, Trichomonas hominis* and *Chilomastix mesneli* from Dr. Di Guisti, Dept. of Preventive Medicine, College of Medicine, New York University; *Tetrahymena gelcii* from Dr. Kessler, College of Physicians and Surgeons, Columbia University; *Leishmania donovani* from the National Institute of Health, Washington, D.C.; *Trypanosoma gambiense* from Dr. D. Weinman, Department of Comparative Pathology and Tropical Medicine, Harvard University Medical School.

[5] Jungeblut, C. W., and Dalldorf, G., *Am. J. Publ. Health,* 1943, **33**, 169; Jungeblut, C. W., *J. Exp. Med.,* 1945, **81**, 275.

TABLE I.

Attempts to Adapt Murine Poliomyelitis Virus to Various Microorganisms by Serial Culture Passages.

Organism	Culture medium with 10% MM virus added	Transfer: Interval days	Transfer: Temp. °C	Presence of virus in serial culture passages: I 10-2	II 10-3	III 10-4	IV 10-5	V 10-6	VI 10-7	VII 10-8	VIII 10-9	IX 10-10	X 10-11*
Mixed bacterial intestinal flora	9.5 cc saline + 0.5 cc 2% bacto-peptone	7	26	—	—	—							
—	" " "	"	"	+	—	—							
B. coli communis	" " "	"	"	?	—	—							
—	" " "	"	"	+	—	—							
Leptospira biflexa	van der Walle's modification of Vervoort-Korthof medium	"	"	+	—	—	—						
—	" " "	"	"	+	—	—							
Entamoeba coli	Egg slant with liver extr. sol. and powdered rice	2	37	+	—	—	—						
—	" " "	"	"	?	—	—	—						
Trichomonas hominis	Egg slant with liver extr. sol.	"	"	+	+	+	+	—	—	—			
"	" " "	"	"	+	+	+	+	—	—	—	—	—	—
" (heat-killed)	" " "	"	"	+	+	+	—	—	—	—	—	—	—
—	" " "	"	"	+	—	—	—	—	—	—			
—	" " "	"	"	+	?	—	—	—	—	—			
Chilomastix mesnili	Cleveland Collins medium and human serum	"	"	?	—	—	—						
—	" " "	"	"	+	—	—	—						
Tetrahymena geleii	2% bactopeptone	7	26	+	+	+	+	—	—	—	—		
"	" " "	2	"	+	+	+	+	?	—	—	—		
"	" " "	"	"	+	+	+	+	?	—	—	—	—	—
—	" " "	7	"	+	+	+	—	—	—	—	—		
—	" " "	2	"	+	+	+	+	—	—	—	—		
—	" " "	"	"	+	+	+	+	?	—	—	—	—	—
Leishmania donovani	NNN medium and Senekji's broth	7	"	+	+	?	—	—					
—	" " "	"	"	+	+	+	—	—					
Trypanosoma gambiense "	Brutsaert & Henrard's modification of van Razgha's medium	"	"	+	+	+	—	—					
—	" " "	"	"	+	+	?	—	—	—				
—	" " "	"	"	+	—								
—	" " "	"	"	+	+	+	—	—	—				

* Theoretical concentrations of virus resulting from dilution.

tent. However, further experiments in which virus was added to a heat-killed *Trichomonas* culture showed that active virus could likewise be carried through 3 serial passages. It would therefore appear that no viral propagation had occurred in these tests and that the presence of the protozoan with its bacterial flora had merely served to render the unfavorable medium more favorable for survival of the originally inoculated virus.

Conclusions. Propagation of human or simian strains of poliomyelitis virus in any kind of culture medium is extremely difficult whereas certain murine strains will grow well in tissue culture containing embryonic mouse brain. A number of enteric bacteria and protozoa as well as some highly differentiated non-intestinal protozoa were examined for their ability to permit propagation of MM murine poliomyelitis virus in culture. No evidence of viral propagation was found as determined by mouse inoculation. The data do not encourage the assumption that neurotropic poliomyelitis virus in stool or sewage may be maintained in symbiosis with free living microbial cells. However the limited extent of this work does not exclude the possibility that experiments with other marine or faecal organisms under different experimental conditions might be more successful.

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Effect of Thiourea on Development of the Sea Urchin Arbacia Punctulata.

GERRIT BEVELANDER.* (Introduced by E. D. Goldsmith.)

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Introduction. The development of the rat,[1-3] the frog,[4-6] and the fish[7] has been influenced by treatment with thiourea. These developmental changes have been attributed to a state of functional hypothyroidism resulting from an interference of thiourea with the production of normal thyroid hormone.[8-10] It has been suggested that the drug may act by inhibiting the peroxidase[11] or the cytochrome oxidase[12] activity of the thyroid gland. From additional studies,[13] however, it appears that thiourea inhibits an oxidative enzyme other than cytochrome oxidase. Recently it has been shown, that low dosages of thiourea incorporated in the food medium produced defects in developing Drosophila,[14]

* With the technical assistance of Miss Myrna Helfman.

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and that slightly higher concentrations were lethal.[15] In view of these facts it became of interest to study the action of thiourea upon the development of the sea urchin egg whose normal embryology is well known.

Material and Methods. The material used in this study consisted of eggs of ripe female sea urchins obtained during the months of July and August. All of the available eggs from each sea urchin were placed in a container and fertilized. The fertilized eggs were transferred to finger bowls containing 200 cc of test solution which was maintained at 21-22.5°C. Each experiment was confined to one egg batch, and several hundred eggs were placed in each of the test solutions. The observations which are to be described below are the results of 25 experiments carried out under the above conditions. Test solutions consisted of 0.1% to 1% of thiourea in sea water. Similar concentrations of urea in sea water, and untreated sea water served as controls.

Cleavage rates and development were ascertained by examining samples of the cultures taken at suitable intervals for periods ranging from 3 to 4 hours for early cleavage and for as long as 72 hours for subsequent development. For the purpose of examining the structure of some of the embryos in greater detail, specimens were fixed, sectioned and stained in the usual manner.

Cleavage and Development. When fertilized sea urchin eggs were exposed to concentrations of 1% thiourea, the eggs as a rule failed to undergo cleavage. In similar concentrations of urea, on the other hand, cleavage proceeded at a relatively normal rate.

In contrast to the effect noted above, when the eggs were exposed to a 0.5% solution of thiourea, early cleavage occurred at a normal rate until the blastula stage was reached. Average results showed that 24 hours after fertilization, the embryos were still in the blastula stage, at 48 hours, the archenteron had usually appeared, but even as long as 72 hours after fertilization, the coelom had not as yet differentiated. In the course of normal development gastrulae develop in about 12 hours and after 24 hours plutei have already differentiated.

Examination of the embryos which had been exposed to 0.5% solutions of thiourea showed that gastrulation had been almost completely inhibited. While the untreated gastrulae attain a size of about 70 μ and then differentiate into plutei, the treated specimens ceased differentiating at the incomplete gastrula stage, but continued to increase in size until they attained a diameter of 90 μ. The ectoderm in these specimens appeared normal, but the primitive mesoderm consisted of a few large cells arranged in irregular patches. Occasionally the first skeletal element, the triradiate spicules, differentiated, but pigmentation of the specimens was as a rule abnormal.

Exposure to concentrations of thiourea ranging from 0.1 to 0.3% did not appreciably affect the cleavage rate. When the eggs were allowed to develop for as long as 72 hours, however, it was observed that there was a progressive decrease in the rate of development of the plutei which was directly proportional to the concentration of the drug.

Inhibition and Recovery. After repeated observations had established the fact that fertilized sea urchin eggs failed to undergo gastrulation in concentrations of 0.5% thiourea, a number of experiments were carried out at this concentration to ascertain more precisely the mechanism responsible for inhibition of gastrulation. Accordingly several egg batches were fertilized and allowed to develop in sea water through the first, second and subsequent cleavages as far as the blastula and gastrula stage. At appropriate intervals several hundred specimens at each of these stages were then placed in a solution of 0.5% thiourea and the subsequent development was observed. In those individuals which had been transferred to the test solution prior to blastulation development proceeded normally until this stage was attained. Gastrulation, however, failed to take place. When swimming blastulae were transferred to the thiourea solutions, gastrulation did not occur. Introduction of well developed gastrulae into thiourea solutions, on the other hand, resulted in the development of plutei at a retarded rate.

Analysis of the data obtained for the ex-

periments described above shows that inhibition of gastrulation does not have its inception in the early cleavage stages and that regardless of previous treatment, thiourea acts selectively upon the blastula to prevent gastrulation, and finally, once gastrulae are differentiated, development as a whole is retarded in 0.5% thiourea, but does nevertheless take place.

In order to ascertain whether the inhibitory effects produced by exposure to thiourea were reversible, several batches of eggs were exposed to concentrations of thiourea ranging from 0.1% to 1% for periods ranging from one hour to 72 hours. They were then washed and returned to sea water. In general, the higher the concentration and the longer the exposure to the drug, the less the recovery, and conversely, with short exposure periods and low concentrations, from 50-90% of the specimens developed into normal plutei after they had been returned to sea water.

Discussion. The observations on the development of thiourea treated sea urchin eggs disclose that exposure to the higher concentrations, 1% or greater, completely inhibit cleavage. Lower concentrations, 0.5%, on the other hand, specifically inhibit gastrulation, while the lowest concentrations found to be effective, 0.1% to 0.3%, produced a retardation in the overall growth rate of the plutei. In the higher concentrations, the inhibitory effects are irreversible, in the intermediate range, reversibility of inhibition takes place to a limited degree, while in the lowest concentrations that were tested, the inhibitory effects are completely reversible.

It is thus apparent that the various physiological processes which operate during cleavage, gastrulation, and growth have different thresholds of inhibition in respect to exposure to thiourea.

Since development is dependent upon the function of a number of enzyme systems and since, further, it has been reported[12,13] that thiourea inhibits certain enzymes which are present in a developing sea urchin egg, it is tentatively suggested that the mechanism by means of which development is inhibited by thiourea in both a general and a selective manner may be most readily accounted for by assuming that thiourea inhibits enzyme systems which are necessary for the differentiation and growth of the sea urchin.

Summary. Sea urchin eggs which were subjected to varying concentrations of thiourea showed a number of pronounced effects. Concentrations of 1% or more resulted in a complete inhibition of cleavage. In 0.5% solutions of thiourea, the eggs developed normally until the blastula stage was reached. For as long as 72 hours, the eggs did not differentiate beyond the gastrula stage. When the arrested gastrulae were returned to sea water, the inhibitory effects were reversible.

In the lowest concentrations used, which ranged from 0.1% to 0.3%, early cleavage was not affected. There was, however, a retardation in the rate at which the plutei developed, which was observed to be proportional to the concentration of the drug.

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Effect of Elevated Body Temperature on Plasma Vitamin A and Carotene.*

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Elevation of the body temperature is well recognized as a therapeutic procedure. "Fever-therapy" is the term now applied to systemic heating of the human body.[1] It has been shown that the therapeutic efficacy of mapharsen, neoarsphenamine, penicillin[2] and the sulfonamides is increased during physically-induced fever. The addition of bismuth enhances the clinical results in the intensive arsenotherapy of early syphilis, although the mode of action has not been well understood.[3] Fever, in combination with these agents (fever-chemotherapy), is a method of intensive treatment applied to syphilis and gonorrhea.[4]

In fever-chemotherapy there are 3 known variables: the height to which the body temperature is elevated, the number of hours this temperature is maintained,[5] and the amount and type of chemotherapy administered.

A number of biochemical studies on body fluids have been made under the influence of physically-induced fever.[6] To our knowledge, no biochemical determination has been found which might serve as an indicator of the intensity of the action of fever-chemotherapy on the human organism. It was believed that a systematic study of the plasma vitamin A level and that of its percursor, carotene, might furnish valuable information concerning the influence of the elevation of body temperature, and serve as a guide in comparing the effects of various combinations of fever-chemotherapy.

A depression of the plasma and serum vitamin A has been found during febrile infectious diseases, especially pneumonia.[7] This

* The authors are greatly indebted to Dr. Herman N. Bundesen, President, Chicago Board of Health, who made this investigation possible and took an inspiring interest in the work. Dr. Theodore J. Bauer, Senior Surgeon, U. S. P. H. S., Venereal Disease Control Officer, cooperated in the organization of this study.

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TABLE I.
Types of Fever-Chemotherapy Studied.

No. patients studied	Clinical diagnosis: Syphilis: Primary	Secondary	Early latent	Chemotherapeutic drugs administered: Before fever	During fever	°F. temp. level	Hr maintained fever
10	2	6	2	150 mg elemental bismuth (bismuth subsalicylate in oil) I.M.	1.76 mg mapharsen per kg body wt.	105.5	8
26	1	14	11	same	same	106	6
21	8	13	0	600,000 O.U. penicillin I.M.	600,000 O.U. penicillin I.M.	106	6
8	2	6	0	same	600,000 O.U. penicillin I.V.	106	3
9	CNS syphilis			none	60 mg mapharsen	105	3
12	Sulfonamide-resistant gonorrhea			8 g sulfathiazole	none	106	8
6	4 "	"	"	sulfathiazole, penicillin or none	"	106	6
	2 gonorrhea relapsing after penicillin						
92	total						

depression of the plasma vitamin A, in all probability, is produced by the retention of vitamin A in the liver.[8] The results of recent investigations indicate that, in general, the regulation of vitamin A levels in the blood is controlled by the liver.[9] Hepatitis, associated with rise of icterus index, may be encountered in intensive fever-chemotherapy.[10]

The purpose of this investigation was to study the influence of the elevation of the body temperature by physical means[4] in combination with various chemotherapeutic drugs on plasma vitamin A and carotene. Blood hemoglobin and total plasma protein determinations were also made to evaluate the possibility of hemo-concentration or dilution during the course of the study.

Experimental Procedures and Results. Studies were made of the effects of 7 different combinations of fever-chemotherapy upon plasma vitamin A and carotene in 92 patients, taken at random from April, 1943 to March, 1945. These treatments and the clinical diagnoses are shown in Table I.

Vitamin A and carotene determinations were made according to Kimble's method with slight modification.[11] The Sheard Sanford Photelometer[12] was employed for quantitative hemoglobin determinations. Specific gravity determinations were performed by the falling drop method of Kagan,[13] from which plasma total protein values were calculated.

The initial values for vitamin A, carotene, total protein of the blood plasma and hemoglobin varied in individual patients over a considerable range (Table II).

A method was adopted to permit statistical and clinical comparisons of the results. The average (arithmetic mean) of 2 or 3 values for vitamin A, carotene, total protein and hemoglobin, found for each individual patient during the 5 days before fever-chemotherapy, was regarded as the base line and made equal to 100. All values found thereafter for the same patient were expressed in % of his prefever (base) value of 100.

On the first and second mornings after 3

[8] Lindquist, T., *Klin. Wchnschr.*, 1937, **16**, 1345, and *Act. Med. Scand. Suppl. No. 97*, 1938.

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[12] Sanford, A. H., Sheard, C., and Osterberg, A. E., *J. Clin. Path.*, 1933, **3**, 405.

[13] Kagan, B. M., *J. Clin. Invest.*, 1938, **17**, 372.

TABLE II.
Maximum and Minimum Values for Plasma Vitamin A, Carotene, Total Plasma Protein and Blood Hemoglobin per 100 cc of 92 Individual Patients before Elevation of Body Temperature, Compared with Values Formerly Obtained and Published by Other Authors.

Patients studied	Vitamin A, I.U.		Carotene, μg		Total protein, g		Hemoglobin, g	
	Max.	Min.	Max.	Min.	Max.	Min.	Max.	Min.
92 (this study)	170	36	163	22	8.25	6.25	17.0	8.6
434 (formerly studied)[11]	189	25	282	28				
(values of other authors quoted from [11])	300	28	347	18				

hours of fever-chemotherapy at 105.0°F (rectal), the plasma vitamin A levels were found to be in about the same range as before treatment in 9 patients.

The patients receiving 3 and 6 hours of fever-chemotherapy at 106.0°F (rectal) or 8 hours at 105.5° to 106.0°F (rectal) showed a decline of the plasma vitamin A and carotene which lasted until the first morning after treatment, except in the 6-hour fever and penicillin group, where a small rise occurred on the first morning after fever. On the second morning after fever, a marked rise of the plasma vitamin A was recorded and, on the third morning after therapy, the plasma vitamin A had returned approximately to the prefever level.

In 3 groups, the medians and the means for vitamin A after fever reached higher values than were recorded before fever. This is similar to the recently reported hypervitaminemia A[14] observed following recovery from infectious febrile diseases.

The changes in the plasma vitamin A were most pronounced in those patients receiving fever-chemotherapy of 8-hour duration. The course of the plasma vitamin A recorded in I.U. per 100 cc plasma for each individual patient in this group is demonstrated in Fig. 1. There are some variations in the extent of these changes but the pattern is practically the same, *i.e.*, a marked decline after fever-chemotherapy, followed by a rapid spontaneous return toward the prefever level.

[14] Steigmann, F., Meyer, K. A., and Popper, H., *Ann. Int. Med.*, 1945, **22**, 832.

For each of the 7 groups of patients who were given different combinations of fever and chemotherapy, the median values and the arithmetic means of plasma vitamin A, carotene, total protein and hemoglobin were calculated. In almost all instances the median values and the arithmetic means were similar. A comparison of these values indicated that the course of the plasma vitamin A and carotene is definitely influenced by the number of hours the body temperature is maintained at 105.0° or 106.0°F (rectal), irrespective of the type of chemotherapeutic drugs given in combination with fever. Therefore, the patients were divided into 3 groups, according to the number of hours the body temperature was elevated to 105.5° or 106.0°F (rectal), to analyze the effect of the duration of fever on plasma vitamin A and carotene.

Group I—Patients receiving 8-hour fever at 105.5° to 106.0°F (rectal).

Group II—Patients receiving 6-hour fever at 106.0°F (rectal).

Group III—Patients receiving 3-hour fever at 106.0°F (rectal).

The median values and the arithmetic means of vitamin A, carotene, total protein and hemoglobin for each of these 3 groups were compiled. Again, both medians and arithmetic means are practically identical. The results for vitamin A, carotene and total protein are demonstrated graphically in Fig. 2.

The results of these studies show that the plasma vitamin A is depressed when the body temperature is elevated to 105.5° or 106.0°F

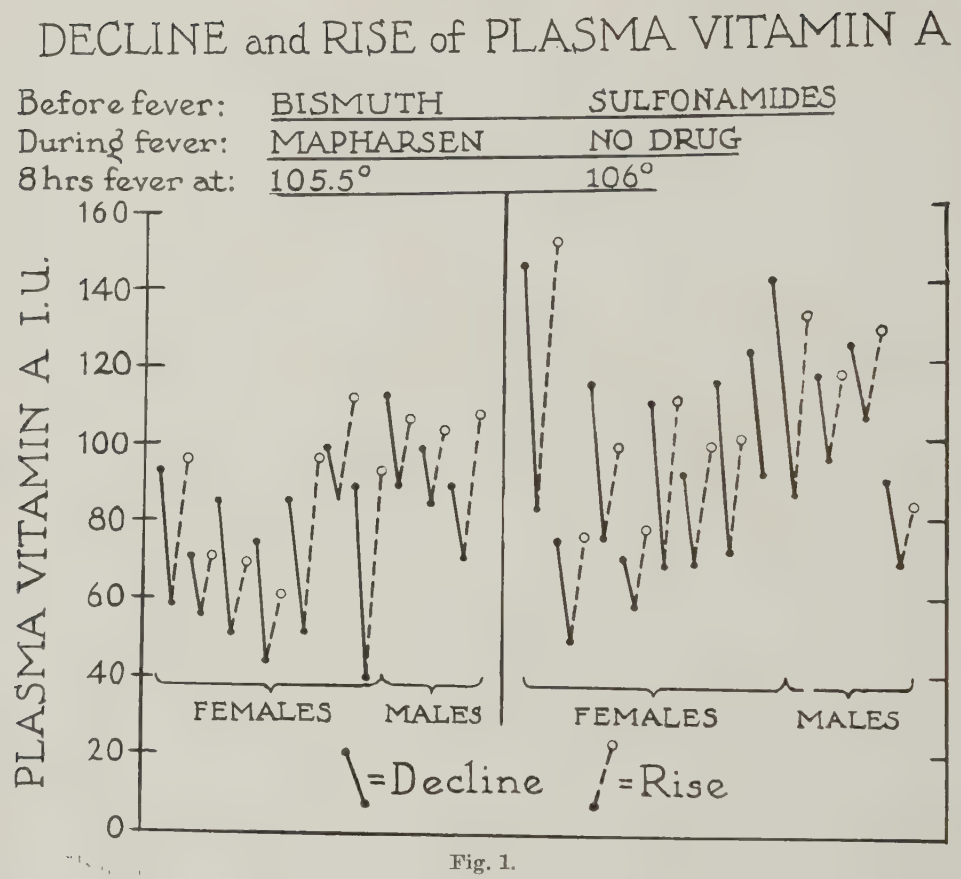

Fig. 1.

(rectal) by means of physically-induced fever. This depression reveals an identical angle of decline irrespective of the length of fever, as may be seen in Fig. 2. The extent of the depression, however, depends on the duration of the fever. The plasma vitamin A drops approximately 30% after 8 hours of fever, 20% after 6 hours, and 10% after 3 hours at 106.0°F (rectal) (Fig. 2). Three hours of fever at 105.0°F (rectal) combined with 60 mg mapharsen had no influence on the plasma vitamin A. The various chemotherapeutic agents, bismuth subsalicylate, mapharsen, sodium penicillin or sulfathiazole, in the amounts administered before or during fever, had no significant influence on the variation of the plasma vitamin A and carotene.

The maximum depression of the plasma vitamin A was noted after termination of fever-chemotherapy. This level remained approximately the same until the next morning, 24 hours following the beginning of the fever session. During the second 24 hours, or from 24 to 48 hours from the time the fever session was begun, the plasma vitamin A level rose, and, in 72 hours from the time the fever was inaugurated, the plasma vitamin A had attained levels which approximated the prefever level. The restoration of the plasma vitamin A occurred spontaneously without any special medication or dietary measures.

The plasma carotene followed a pattern similar to that of vitamin A, although there was less depression of the carotene, *viz.*, approximately 20% after 8 hours, 15% after 6 hours, and 10% after 3 hours of fever (Fig. 2). The restoration of the plasma carotene level was similar to the plasma vitamin A.

Hydremia, as evidenced by the decrease in total plasma protein and hemoglobin levels,

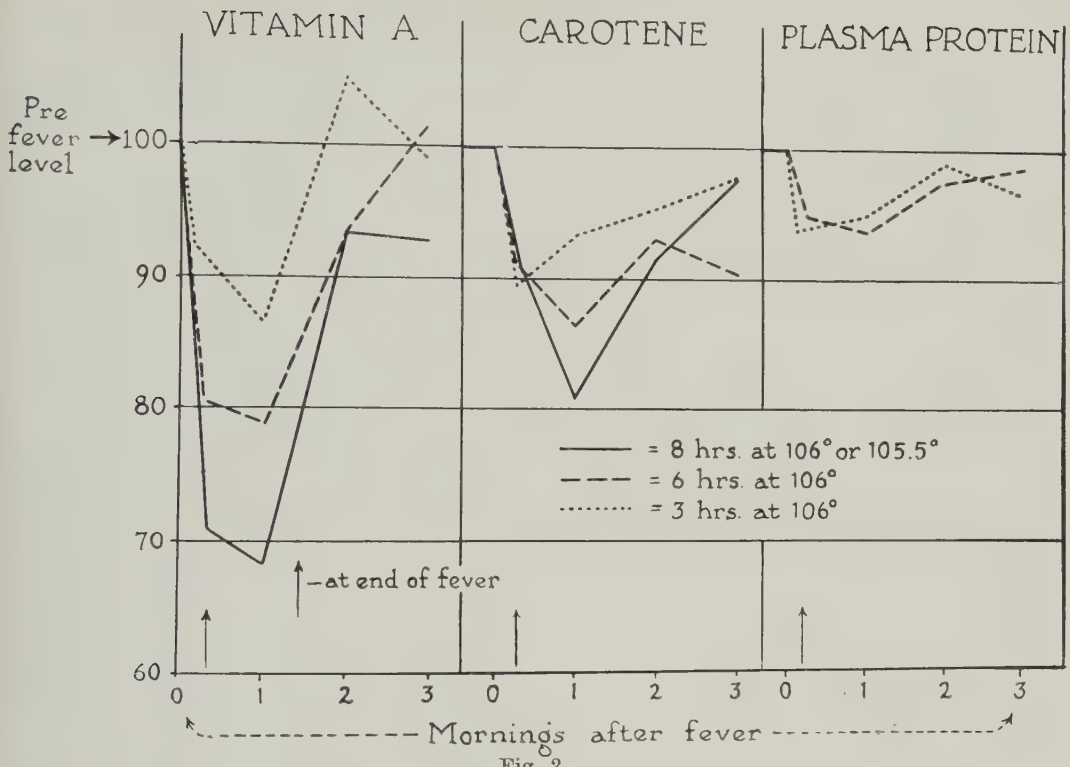

Fig. 2.

was present but not of sufficient magnitude to account for the decreases in the plasma vitamin A and carotene levels. This is particularly illustrated in the 8- and 6-hour fever-chemotherapy groups where the decrease in the levels was from 3 to 7 times that of the hemoglobin or total plasma protein. Fig. 2 shows the changes in plasma protein. The changes in hemoglobin are parallel.

Each of the 22 patients given 8-hour sessions of fever-chemotherapy showed a depression of the plasma vitamin A of more than 15%. A total of 53 patients in the 3 groups were given 6-hour sessions of fever-chemotherapy at the level of 106.0°F (rectal), and all but one patient showed decreases of the plasma vitamin A level at the termination of the fever sessions or on the first morning after fever, as compared to the prefever levels. Five of these patients did have a depression of less than 10%, *i.e.*, within the possible range that might be attributed to the error of the method, for which no clinical explanation can be given. A depression of the plasma vitamin A level was found in all of the 8 patients subjected to 3-hour fever-chemotherapy at 106.0°F (rectal). One of these patients had a depression of less than 10%. The number of hours the fever was maintained to some degree may account for this factor, as this phenomenon was not observed in the 8-hour fever sessions.

There was no significant difference in the vitamin A levels of those patients who experienced uneventful fever sessions as compared to those who had clinical evidence of dehydration, emesis or low blood pressure during or after fever-chemotherapy.

Four of the 84 patients who received fever-chemotherapy at the level of 105.5° to 106.0°F (rectal) developed mild jaundice with an icterus index† between 14 and 45 units. The plasma vitamin A values of 3 of these pa-

† Icterus index (I.I.) was determined by comparison of the color of the serum against a standard solution of potassium dichromate in a block comparator.[15]

[15] Simmons, J. H., and Gentzkow, C. J., *Laboratory Methods of the U. S. Army, 5th Ed.*, 1944, 71.

tients compared to the prefever levels were definitely lower than those of any patient in the same group without jaundice.

There was a delay, however, in the spontaneous rise of the plasma vitamin A on the second and third days after fever in those patients who developed jaundice. The reports of others[16] and our own observations point toward the idea that the "recovery rise" of the plasma vitamin A after fever may be an indicator of the return of normal liver function. As long as the liver function is impaired, insufficient vitamin A is released from the liver stores into the circulation. On this basis, therefore, the plasma vitamin A may remain at a low level. Vitamin A is released into the blood stream when the liver regains its normal function and then the plasma vitamin A increases. In those patients in whom the rise of plasma vitamin A was delayed, after fever-chemotherapy clinical jaundice and an elevated icterus index were noted simultaneously with the delay or soon thereafter.

These observations suggest that if the plasma vitamin A is depressed to 50% or less of the prefever level, the development of jaundice may be expected. However, the reverse did not hold true as elevated icterus indices were not necessarily concurrent with extremely low plasma vitamin A values.

Summary. In a study of 92 patients it was found that elevation of the body temperature to 105.5° or 106.0°F (rectal) by physically induced fever was followed by a depression of the plasma vitamin A and carotene. The extent of the depression was directly related to the duration of the fever. The course of the plasma vitamin A and carotene was almost identical irrespective of the chemotherapeutic agents (bismuth subsalicylate in oil, mapharsen, sulfathiazole or the sodium salt of penicillin) given either before or during physically-induced fever of equal duration. It is, therefore, justifiable to conclude that elevation of the body temperature is the principal cause of the depression of plasma vitamin A and carotene. At the termination of fever, the plasma vitamin A was nearly at the lowest level. The restoration of the plasma vitamin A level usually occurred by the second day after treatment and took place spontaneously without any special medication or dietary measures. The plasma carotene level showed a pattern similar to that of the plasma vitamin A. The depression of the carotene was generally not as great and occurred more slowly. This behavior of the plasma vitamin A is, to the best of our knowledge, the first biochemical indicator shown to serve as a measurement of the intensity of the action of physically-induced fever on the human organism.

[16] Meyer, K. A., Steigmann, F., Popper, H., and Walters, W. H., *Arch. Surg.*, 1943, **47**, 26.

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Demonstration of Infectious Hepatitis Virus in Presymptomatic Period after Transfer by Transfusion.*

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The present paper is to report observations upon the presence of the virus of infectious hepatitis in the serum of a patient following the receiving of a transfusion of blood taken from another individual 48 hours before the onset in the latter of apparently naturally acquired infectious hepatitis.

The circumstances surrounding this series

* This investigation was conducted under the Commission on Influenza, Board for the Investigation and Control of Influenza and other Epidemic Diseases in the Army, Preventive Medicine Service, Office of the Surgeon General, United States Army.

of events were briefly as follows:

Sgt. L. F. was a member of a general hospital medical detachment who had been in consistently good health.

May 8, 1944 Played baseball with vigor.
" 9 Donated 500 cc of blood which was given to patient McG. on May 10.
" 11 L. F. reported to sick call. Questionable icterus of sclerae was reported and he was admitted to hospital.
" 12 Mild jaundice was present.
" 13 Appeared to be average case of infectious hepatitis but during night became actively delirious.
" 14 Became comatose and expired.

This patient appears to represent an example of naturally acquired infectious hepatitis in which the onset and the appearance of jaundice coincided. The course was unusually brief with fatal outcome.

Pfc. McG. was admitted to a hospital in Italy April 25, 1944, because of perforating wound of abdominal wall.

May 3, 1944 Transferred to a general hospital in the base area. Progress had been satisfactory.
" 10 Received transfusion of blood from L. F.
" 18 Serum 5-187 used in this study was obtained.
" 19 A series of tests revealed no abnormality.
" 21 Complained of lower abdominal pain and discomfort; one loose stool; temp. 99°.
" 23 Enlargement of cervical lymph nodes.
" 24 Serum 5-231 used in this study was obtained.
" 25 Occasional needle-like pains in the right upper quadrant. Liver just palpable and very tender.
" 27 Liver larger, tender; tip of spleen palpable. Patient felt better.
" 29 Patient felt well. Liver smaller and less tender.
" 30 Frontal headache, temp. 102.6°. Liver edge one f.b. below costal region; not tender.
" 31 Patient felt ill; icteric tinge to sclerae.
June 1 Definite icterus. Patient quite ill. Temp. 98° to 102.6°. Liver larger and very tender.

There had been no history of malaria, previous jaundice or dysentery, no known insect bites. Malarial smear and Kahn test were negative; no ova or parasites were found in stool.

The patient's course was that of a seriously ill person for one week thereafter, but recovery was satisfactory. It seems quite certain that transfusion was the method by which the virus was transmitted, with symptoms 11 days and jaundice 21 days, thereafter.

Materials. The histories of the cases and the materials employed in the study were obtained by Colonel M. H. Barker, M.C., AUS., and Captain Frederick G. Robbins, M.C., AUS., of the 15th Medical General Laboratory in Italy (Colonel V. H. Cornell, M.C., AUS., Commanding). The sera were stored in dry ice and shipped in a frozen state by air to Brigadier General S. Bayne-Jones, Preventive Medicine Service, Office of The Surgeon General, United States Army, but were in a liquid state at the time of their arrival. They were again frozen in CO_2 ice and transferred, frozen, to this laboratory where they were stored in CO_2 ice until preparation for study.

Subjects. The subjects were inmates of the State Prison of Southern Michigan, Jackson, Michigan, who volunteered to participate in the study. Only one case of catarrhal jaundice had been seen in the institution in a period of 9-10 months. From those who offered to take part, the subjects were selected after thorough physical examination. X-ray of the chest was taken in each instance to eliminate those with significant pulmonary or cardiac abnormalities. Examinations of the urine included those for urobilinogen and bilirubin; examination of the blood was made for bilirubinemia. Bromsulfalein retention (5 mg—30 minutes) and Hanger cephalin-cholesterol flocculation tests[1,2] were carried out for evidence of hepatic dysfunction. Individuals with history of syphilis, positive

[1] Hanger, F. M., *J. Clin. Inv.*, 1939, **28**, 261.
[2] Frisch, A. W., and Quilligan, J. J., in press.

TABLE I.
Results of Inoculation of Volunteers with Serum from Infectious Hepatitis.

Group	Subject	Age	Race	Inoculated	Onset of: First possible symptoms days	Onset of: Definite illness days	Onset of: Jaundice days	Laboratory evidence	Duration days
1	Fd	24	C	4/13/45	38	38	42	+	8
	Fy	24	W	"	38	40	45	+	21
	Mc	24	W	"	35	43	47	+	34
	Ro	34	W	"	0	0	0	0	0
2	Bn	21	W	4/13/45	42	0	0	0	5
	Dn	22	C	"	*	0	0	0	—
	Gy	25	W	"	45	0	0	0	6
	Lr	30	W	"	43	43	46	+	35

* This patient complained of headache, nausea, vomiting and tenderness in right upper quadrant intermittently from the second day after inoculation for a period of 3 months. Certain laboratory tests were irregularly suggestive between the fifth and ninth weeks after inoculation but neither clinical nor chemical evidence of icterus was obtained.

serological tests or history of previous attacks of jaundice were excluded. Only one of the selected individuals was over 30 years of age.

Inoculations. (1) Serum 5-187 was obtained May 18, 1944, from patient McG. 8 days after receiving transfusion, 3 days before any symptoms and 13 days before icterus was noted. The serum had been preserved in a tightly stoppered tube by freezing in a CO_2 ice cabinet. On April 10, 1945, the specimen was thawed; 1.25 cc of serum was mixed with 3.75 cc of sterile Ringer solution, centrifuged at 1500 r.p.m. for 15 minutes and filtered through a Mandler filter under 9-lb. pressure per square inch. Aerobic and anaerobic cultures were bacteriologically sterile after 48 hours. On April 13, 1945, 1.0 cc of the filtrate of the 1:4 dilution of serum was given subcutaneously to each of 4 volunteers (Fd, Fy, Mc, Ro) with care taken to use individual needles and syringes.

(2) The same procedure was followed with the sample of serum 5-231 obtained from patient McG. May 24, 1944, 14 days after transfusion, 3 days after the first of any symptoms, and 7 days before jaundice was clinically detected. On April 13, 1945, 1.0 cc of the filtrate of the 1:4 dilution of serum was given subcutaneously to each of 4 volunteers (Bn, Dn, Gy, Lr) with individual needles and syringes.

Observation. The inoculated individuals were returned to their usual quarters and permitted to continue their usual activities, including customary work, until any evidence of illness was obtained. During this period they were examined twice weekly by one of the investigators and temperatures and symptoms were recorded. Examination of the urine, including tests for urobilinogen and bilirubin; leukocyte counts, blood bilirubin, bromsulfalein and cephalin-cholesterol flocculation tests were done at 10-day intervals. When any suggestive symptoms or signs were noted the patient was transferred to the institutional hospital where close clinical observations could be maintained. There laboratory examinations were frequently made (with the exception of bromsulfalein) and diets high in carbohydrate and selected protein were instituted. Glucose, sucrose, amino acid concentrates, plasma, vitamin B complex and other fluids were given intravenously in an effort to minimize the course of the disease. All individuals were kept under observation for at least 130 days.

Results. In Table I are summarized the results observed in the 2 groups of individuals. Three of the 4 inoculated subcutaneously with serum from McG. 8 days after transfusion developed infectious hepatitis and jaundice. The first symptoms which seemed even in retrospect to be of significant relationship were noted 35, 38 and 38 days, respectively, after inoculation. In only the case of Mc was there an appreciable interval, 8 days, between the first symptoms noted and what appeared to be the actual

onset of the preicteric stage of clinical illness. The latter figures were 38, 40 and 43 days, respectively, after inoculation while clinical icterus was noted after intervals of 42, 45 and 47 days. Biochemical tests were correspondingly positive. The marked variation in clinical severity in the 4 individuals receiving the same inoculum was striking. In one, Fd, a rapidly developing disease with fatal termination was encountered; another, Mc, had a prolonged course with a brief relapse; another had quite a mild clinical disease with maintenance of good physical state and good appetite most of the time; the fourth had no detectable disturbance.

One of the second group of subjects, inoculated with serum obtained from the same patient 3 days after the first minimal symptoms and 6 days after the specimen used with group 1, developed symptoms 43 days, and jaundice 46 days, after injection. At the time the other patients were developing hepatitis the 3 additional members of this group had short periods of gastric distress, lassitude, tenderness in right upper quadrant; no jaundice developed and in only one of the 3, Dn, was any disturbance of liver function suggested by laboratory procedures.

Although the subjects were not isolated during the period before the onset of illness no other cases of jaundice occurred in the population.

Discussion. The results of the above observations are of interest from several points of view. It seems quite clear that the disease in the original donor of blood was naturally acquired infectious hepatitis which was transmitted from him by transfusion of blood before the recognized onset of his illness. In turn, the agent was demonstrated in the blood of the recipient 3 days before the onset of any symptoms while biochemical tests were entirely normal, 13 days before the onset of jaundice. The first symptoms noted in the recipient of the transfusion were 11 days after the transfusion and jaundice began 21 days after transfusion.

Both Havens,[3] and Neefe, Stokes and Gellis,[4] have commented that with serum from cases of naturally acquired infectious hepatitis the interval between administration and onset of jaundice was less than 37 days regardless of whether the material was given by mouth or parenterally. Oliphant,[5] however, employed for subcutaneous inoculation 0.5 cc of a 1:6 dilution of serum from a case of infectious hepatitis naturally acquired in Italy and induced jaundice in 4 of 11 subjects after intervals of 85-106 days. MacCallum and Bradley[6] also witnessed incubation periods of 64, 75 and 92 days after subcutaneous inoculation of 1.25 cc of serum from infectious hepatitis. In the present study the average interval before jaundice in the 4 volunteers receiving the equivalent of 0.25 cc of filtered serum subcutaneously was 45 days, a length of time intermediate between the 2 groups of observations.

Havens[7] states that no appreciable difference in incubation period was observed when doses of 0.01 to 0.5 cc of the same serum from infectious hepatitis was given subcutaneously. Neefe, Stokes, Reinhold and Lukens[8] gave 1, 9, 10 and 12 cc, respectively, of icterogenic plasma intravenously to 4 subjects and jaundice occurred at decreasing intervals of 110, 99, 74 and 73 days, thereafter, suggesting some relation to the amount injected. A fifth subject who received 100 cc did not develop jaundice. In the present study the average incubation period was more than twice as long in the volunteers (third passage) as in the transfused patient (second passage) from whom the serum was obtained, although the route was in both instances parenteral. Moreover, there was a relative uniformity in the time of onset of jaundice in the inoculated individuals although the

[3] Havens, W. P., Jr., PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 203.

[4] Neefe, J. R., Stokes, J., Jr., and Gellis, S. S., *Am. J. Med. Sci.*, 1945, **210**, 561.

[5] Oliphant, J. W., *Pub. Health Rep.*, 1944, **59**, 1614.

[6] MacCallum, F. O., and Bradley, W. H., *Lancet*, 1944, **2**, 228.

[7] Havens, W. P., Jr., *Am. J. Pub. Health*, 1945, **36**, 37.

[8] Neefe, J. R., Stokes, J., Jr., Reinhold, J. G., and Lukens, F. D. W., *Am. J. Clin. Inv.*, 1944, **23**, 836.

clinical responses varied considerably in severity. The difference strongly suggests that the amount of effective virus in the inoculum had a significant influence upon the result. If this should not be the case one might suggest that a variation in the virus had occurred during its 2 passages by parenteral inoculation in human subjects. In any case the data illustrate the difficulty in classifying infectious hepatitis on the basis of the incubation period after parenteral injection of icterogenic serum. The relative uniformity in the time of onset of jaundice in the inoculated individuals indicates, on the other hand, that the variation in severity of the clinical courses following the same inoculum is related to individual susceptibility.

The events demonstrate how, with virus in the blood well before the onset of symptoms, infectious hepatitis may be transmitted by blood products derived from individuals in whom the presence of the virus would be entirely unsuspected.

Summary. A transfusion of blood from an individual in the incipient stage of infectious hepatitis elicited the first symptoms in the recipient after 11 days and jaundice after 21 days.

By subcutaneous inoculation of human volunteers the virus was demonstrated in the serum of the recipient on the eighth day after transfusion which was 3 days before the first symptoms and 13 days before the onset of jaundice. Virus was also demonstrated in another specimen of serum taken 6 days later, or 3 days after first symptoms.

The incubation period as measured by the onset of jaundice in the 4 subjects who had definite illness was 42 to 47 days, while the clinical response varied greatly in severity.

The significance of the results is discussed.

This investigation was made possible by the cooperation of Dr. Garrett Heyns, Director of Corrections, State of Michigan, and Dr. W. B. Huntley, Medical Director of the State Prison of Southern Michigan, Jackson, Michigan, and his staff, to whom the authors wish to express their appreciation for constant assistance. They wish also to express their indebtedness to the volunteers who participated so willingly and cooperatively in the study.

15300

Sources of Energy for Intestinal Smooth Muscle Contraction.*†

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The rhythmic contractions of a segment of rabbit small intestine, suspended in glucose-free Tyrode solution, were found by Rona and Neukirch[1,2,3] to undergo a gradual and progressive decrease in amplitude; at which time, the addition of certain substances (glucose, mannose, pyruvic, lactic, acetic, butyric, β-hydroxybutyric and oxaloacetic acids) served to increase and maintain the amplitude of contraction, the extent of the increase varying with the substance added. They attributed the progressive decrease in the height of the contractions in the absence of glucose to gradual depletion of the endogenous stores

* The work described in this paper was done under a contract recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Cornell University Medical College.

† We are indebted to Dr. David Rittenberg for samples of enanthic and pelargonic acids, and to Dr. Severo Ochoa for the α-ketoglutaric acid used in this study.

1 Neukirch, P., and Rona, P., *Arch. f. Physiol. (Pflüger's)*, 1912, **144**, 555.

2 Rona, P., and Neukirch, P., *Arch. f. Physiol. (Pflüger's)*, 1912, **146**, 371.

3 Rona, P., and Neukirch, P., *Arch. f. Physiol. (Pflüger's)*, 1912, **148**, 273.

of energy-yielding substrates. The effectiveness of a substance in restoring amplitude was considered a measure of its ability to serve as a source of energy for smooth muscle contraction. More recently, Feldberg and Solandt,[4] and Feldberg,[5] in extending the work of the earlier investigators, have arrived at the same conclusions.

Except for the studies cited, the procedure introduced by Rona and Neukirch has not been utilized for the investigation of the intermediary metabolism of smooth muscle. If their assumption is correct, that the restoration of amplitude is evidence of the ability of a substance to provide energy for smooth muscle contraction, then the method would appear to be unusually well suited for such studies. The restoration of contractility would comprise a more exacting and reliable criterion for the evaluation of a substrate, than is provided by a rise in oxygen consumption alone. The latter criterion is frequently undependable; the former furnishes direct evidence that the energy provided by a substance under study is actually utilizable for smooth muscle contraction.

In the present study the method has been employed for a more extensive investigation of potential energy-yielding substrates, selected on the basis of modern concepts of intermediary metabolism, and particular attention has been given to the possible usefulness of fatty acids.

Methods. The standard preparation consisted of a fresh segment, approximately 4 cm long, from the upper portion of rabbit small intestine. The segment, with its lumen open, was suspended in a 50 cc muscle chamber, and attached to an isotonic muscle lever. The lever exerted a tension of 4 g, and recorded the contractions of the longitudinal muscle on a kymograph. The intestinal segment was bathed in glucose-free Krebs-Henseleit solution,[6] maintained at 37.5°, and aerated with 95% O_2 - 5% CO_2 to obtain a pH of 7.4.

Prior to testing each substance, the spontaneous rhythmic contractions of the longitudinal smooth muscle were allowed to decrease to about 1/10 to 1/20 of the original amplitude. This usually required less than 1 hour and introduced no change in contraction frequency. At this point, the test substance, dissolved in a small quantity of Krebs solution, was added to the muscle chamber. All acids were used in the form of sodium salts. For each substance tested, the minimal concentration was determined which brought about the maximal amplitude of contraction of which that substance was capable. This increase in amplitude was then compared on a percentage basis with the maximal change which resulted from the addition of glucose to the same intestinal preparation.

Results. The record of a typical experiment is reproduced in Fig. 1. It illustrates the gradual decrease in amplitude and average tonus which takes place in the absence of substrate as well as the restoration of both on the addition of glucose (200 mg %) when the amplitude had fallen to about 1/10 to 1/20 of the original value. The amplitude and average tonus began to increase within a minute of the addition of glucose, and the maximal amplitude was reached within 10 minutes, and maintained thereafter. In this experiment, the maximal amplitude following glucose was greater than that initially exhibited; in other experiments, the amplitude attained after the addition of glucose was either equal to, or greater than, but never less than, the original amplitude. Not discernible in the tracing because of the reduction in size, are the number of contractions per minute given by the intestinal segment and the constancy of this frequency throughout the experiment, despite marked changes in amplitude. Under our experimental conditions, the contractions averaged 17 per minute with most of the intestinal segments used. This uniformity of contraction rate throughout the experiment simplified the comparison of the relative effectiveness of different substrates in providing energy for contraction, since the only variable introduced consisted of a change in amplitude.

[4] Feldberg, W., and Solandt, O. M., *J. Physiol.*, 1942, **101**, 137.

[5] Feldberg, W., *J. Physiol.*, 1943, **102**, 108.

[6] Krebs, H. A., and Henseleit, K., *Z. f. Physiol. Chem.*, 1932, **210**, 33.

Fig. 1.

Kymograph tracing showing decrease in amplitude of intestinal contractions in glucose-free solution, and restoration of contraction amplitude on addition of glucose (at arrow) to provide a concentration of 200 mg %.

The second step in the experimental procedure—that is, the comparison of the relative effectiveness of different substrates in supporting smooth muscle contractions—is illustrated in Fig. 2. The results obtained with 4 substrates are expressed in semi-diagrammatic fashion, each contraction wave representing approximately 17 contraction waves in the actual kymograph tracing. With the amplitude after addition of glucose as the standard of reference, the relative effectiveness of the other substrates is as follows: pyruvic acid, 100%; butyric acid, 50%; and enanthic acid, 20%.

In Tables I and II, are assembled the results obtained with a number of substances which proved capable of restoring, in varying degrees, the amplitude of smooth muscle contraction. In Table I, the height of the response is compared with that produced by glucose, and, for each substance, represents the maximal response which it can elicit. Table II lists the minimal concentration of each substrate required to bring about its individual maximal increase in amplitude.

Table III lists those substances which failed

Fig. 2.

Semi-diagrammatic representation, based on kymograph tracings, illustrating relative effectiveness of glucose, pyruvic, butyric, and enanthic acids in restoring amplitude of contraction of intestinal smooth muscle after prior exposure to a glucose-free solution.

TABLE I.
Substances which Increase Amplitude of Contraction.

Substance	Response compared with that of glucose
	%
Glucose	100
Mannose	70-90
Acetic acid	100
Pyruvic acid	100-110
Oxaloacetic acid	75-100
d,l-Lactic acid	20-40
Acetoacetic acid	20-30
Butyric acid	40-60
Caproic acid	40-60
Caprylic acid	40-60
Pelargonic acid	35-45
Enanthic acid	15-20
Valeric acid	5-10

to increase the amplitude of contraction. Each was tested over a range of concentration extending from 10 to 100 mg %, and found to be without influence on the gradual reduction in tone and amplitude of contraction characteristic of the preparation in the absence of substrate. There were 2 exceptions: acetaldehyde and glyceraldehyde, in concentrations of 100 mg %, exerted a marked inhibitory effect, as evidenced by the rapid and virtually complete cessation of all contractile activity. The inhibition induced by acetaldehyde could not be overcome by the subsequent addition of glucose, pyruvate, acetate or lactate, whereas that caused by glyceraldehyde could be overcome by pyruvate, acetate, or any of the fatty acids listed in Table I, but not by glucose or lactate. In the case of citric acid, which removes Ca ions from solution to form a weakly dissociable

TABLE II.
Minimal Concentrations Necessary for Maximal Increases in Amplitude of Contraction.

Substance	Minimal concentration in mg %
Glucose	100-125
Mannose	125-150
d,l-Lactic acid	20-30
Oxaloacetic acid	20-30
Acetic acid	10-15
Pyruvic acid	10-15
Butyric acid	4-5
Valeric acid	3-4
Caproic acid	2-3
Enanthic acid	1-2
Caprylic acid	0.5-0.75
Pelargonic acid	0.5

TABLE III.
Substances which Fail to Increase Amplitude of Contraction.

Galactose	Propionic acid
Fructose	Hydroxyacetic acid
Glycogen	Citric acid
Glucose-1-phosphate	Succinic acid
β-Phosphoglyceric acid	Fumaric acid
Glycerol	α-Ketoglutaric acid
d,l-Glyceraldehyde	Glycine
Acetaldehyde	1(+)-Alanine
Ethanol	1(—)-Aspartic acid
Acetone	1(+)-Glutamic acid

$(Ca\ citrate)^-$ complex, the precaution was taken to add sufficient Ca ions (as $CaCl_2$) to replace those removed, in order to avoid changes in muscle tonus which would otherwise have resulted from a deficiency of Ca ions in the medium.

Fig. 3 is a graphic presentation of the maximal increases in amplitude observed with the series of fatty acids from acetic through pelargonic. The greatest increase, which equalled that given by glucose, was obtained with acetic acid. The remaining even-carbon fatty acids (butyric, caproic and caprylic) all produced an identical increase in amplitude, which was approximately 50% of that of acetic acid. On the other hand, the effects of the odd-carbon series of fatty acids were strikingly different. Propionic acid was devoid of effect. The changes in amplitude induced by the other members of the odd-carbon series were less (valeric, enanthic and pelargonic) than those obtained with even-carbon fatty acids, and differed in extent with each compound. The magnitude of response increased as the chain-length increased. Thus, the approximate increase with valeric acid was 10%, with enanthic acid 20%, and with pelargonic acid 40% of that observed with acetic acid.

Experiments of a preliminary character have been carried out to investigate the mechanisms responsible for the differences exhibited by odd- and even-carbon fatty acids. If β-oxidation takes place in smooth muscle, as our experiments suggest, the smaller fragments resulting from this type of degradation of the even-carbon fatty acids would all be utilizable for muscle contraction; whereas the odd-carbon fatty acids would leave a non-utilizable propionic acid residue. Since the

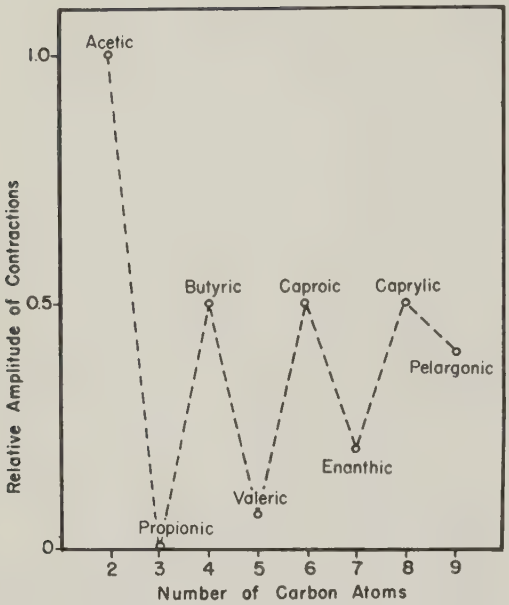

Fig. 3.
Relative effectiveness of odd- and even-carbon fatty acids in restoring amplitude of contraction of intestinal smooth muscle following prior exposure to glucose-free solution.

propionic residue would be formed at the surface of the postulated β-oxidation enzyme, it might conceivably compete for this surface with the unaltered fatty acid or its oxidizable fragments. This concept was explored by adding propionic acid in concentrations of 10-30 mg % to muscle preparations contracting under the influence of butyric or caprylic acid, in concentrations of 5.0 and 0.75 mg % respectively. In each instance, 30 mg % of propionic acid served to reduce the amplitude of contraction by about 80%. This inhibition, however, could subsequently be overcome by doubling the concentrations of butyric or caprylic acid.

The observation was also made that relatively high concentrations of enanthic, caprylic and pelargonic acids completely inhibited smooth muscle contraction. The effective concentrations varied with different intestinal preparations and were as follows: enanthic 50-200 mg %; caprylic 25-150 mg %; and pelargonic 20 mg % (1 experiment). This inhibition was not overcome by the addition of glucose, pyruvic acid, or acetic acid. However, it proved to be a reversible inhibition, since contractility was restored when the fatty acid was washed out and replaced with fresh Krebs solution.

Discussion. 1. *Mode of Action of Substances Which Restore Contractility.* Those substances which restore contraction amplitude may act either as substrates or pharmacological agents. All the evidence so far accumulated, although indirect, appears to favor the substrate concept. Rona and Neukirch[1] observed a reduction in glucose and mannose content during a 2½-hour period of active contractions. More specific evidence against a pharmacological action was provided by Feldberg and Solandt.[4] The addition of acetylcholine during a period of diminished activity in a glucose-free Tyrode solution produced a relatively small increase in tonus, sustained for less than a minute. However, following the restoration of amplitude by glucose or pyruvate, acetylcholine produced a marked and well-sustained increase in tonus. From this it was inferred that glucose and pyruvate do not increase smooth muscle activity by increasing the production of acetylcholine, and that this agent could exert its pharmacological effect only when energy for contraction was provided by appropriate substrates. These experiments have been confirmed and extended in our laboratory, with the observation that large and sustained increases in tonus may also be obtained with acetylcholine after the restoration of amplitude by fatty acids, such as acetate and butyrate. Although this evidence is indirect, we favor the substrate concept over the pharmacological, particularly in view of the conformity of much of the data obtained with prevailing views of intermediary metabolism. The discussion of our results has therefore been based on the concept that the capacity of a substance to augment and sustain contraction amplitude is derived from its ability to provide energy for smooth muscle contraction.

2. *Carbohydrate Series.* Of the hexoses studied, only glucose and mannose yielded energy for contraction; galactose and fructose were ineffective. In 2 respects our findings differ from those of Feldberg and Solandt.[4] We failed to find the increase in reactivity to acetylcholine reported by these investiga-

tors to follow the addition of fructose to a glucose-free medium. We also found mannose to be 70-90% as effective as glucose, in contrast with the 25-30% effectiveness which they observed. This latter discrepancy may be due to the fact that they did not use maximal increases in amplitude as the basis for comparison.

The excellent substrate effect of pyruvic acid is in accord with the Meyerhof cycle. The smaller effect produced by lactate is of interest. The lack of effectiveness of glycogen, glucose-1-phosphate, and β-phosphoglyceric acid need not be considered evidence against the occurrence of the Meyerhof cycle in smooth muscle since it is questionable whether the muscle cells are permeable to these substances. The same reservation holds for the ineffectiveness of glucose-6-phosphate and fructose-1,6-diphosphate, reported by Feldberg.[5]

3. *Acetic Acid.* This proved to be an excellent substrate, producing contraction amplitudes equal to glucose and pyruvate. This is compatible with recent observations on the utilization of acetate by a variety of tissues. The pathway for the oxidation of acetic acid in smooth muscle remains obscure; it does not appear to be oxidized to hydroxyacetic acid, since the latter had no substrate effect.

4. *The Krebs Tricarboxylic Acid Cycle.* Of the acids postulated in this cycle the following were tested and found ineffective: citric, α-ketoglutaric, succinic and fumaric. Oxaloacetic acid exerted a good substrate effect. However, the exceptional behaviour of oxaloacetic acid prompts the suggestion that it may have exerted its effect only after decarboxylation to the readily oxidizable pyruvic acid. The ineffectiveness of succinic acid is in accord with *in vitro* metabolic experiments with strips of dog intestinal smooth muscle (unpublished data). In these experiments carried out at reduced O_2 tensions, the increased O_2 uptake in the presence of succinic acid failed to increase the concentrations of phosphocreatine and adenylpyrophosphate, the high energy phosphates essential for muscle contraction. These results provide no support for the participation of the postulated tricarboxylic acid cycle in the activity metabolism of smooth muscle, and, by the same token, would exclude the cycle as a pathway for the oxidation of pyruvic and acetic acids.

5. *Amino Acids.* In conformity with the earlier observations of Neukirch and Rona,[2] the amino acids studied were found to exert no substrate activity. This was of particular interest with respect to alanine and aspartic acid, since their corresponding keto-acids, pyruvic and oxaloacetic, are extremely effective. Apparently, intestinal smooth muscle is unable to deaminate these amino acids to their keto forms.

6. *Ketone Bodies.* Neukirch and Rona[2] had previously reported a small increase in amplitude with β-hydroxybutyric acid. We have observed an effect of similar magnitude with acetoacetic acid, but none with acetone. Should the lack of utilization of acetone prove to be a general phenomenon, it might provide an explanation for the readiness with which acetone appears in the urine even with mild disturbances in fat metabolism.

7. *Fatty Acids.* The difference in the effects observed with odd- and even-carbon fatty acids strongly suggests that β-oxidation takes place in intestinal smooth muscle. Thus, butyric, caproic and caprylic, which can all be completely degraded to 2-carbon fragments, are all equally effective as substrates. On the other hand, propionic acid, which is not susceptible of β-oxidation, is ineffective; and the remaining odd-carbon fatty acids studied, all of which would leave a propionic acid residue on β-oxidation, have a smaller effect than those of the even-carbon series.

An explanation for the identical effects of the even-carbon fatty acids may be that they all undergo β-oxidation at the same enzyme surface; and that, once this surface is saturated with any one of them, β-oxidation then proceeds at the same rate. Support for this hypothesis is provided by the fact that once a maximal increase in amplitude is induced by any one of the series, the subsequent addition of another results in no further increase.

Our experimental results also permit a tentative explanation for the smaller effects of the odd-carbon acids (valeric, enanthic, pelargonic) as well as for the increase in

amplitude with the increase in chain-length. The β-oxidation of odd-carbon fatty acids should in every instance leave a non-utilizable propionic acid residue. Propionic acid has been found to reduce the effectiveness of even-carbon fatty acids as substrates, an inhibition which can be overcome by raising the concentration of the utilizable fatty acid. This suggests the competition of propionic acid for the surface of the β-oxidation enzyme. With the odd-carbon series, the competition between the propionic residue and the unaltered acid and its β-oxidizable fragments, would explain the smaller effects of this series. This competition should become less effective as the chain-length increased, since the β-oxidizable portion would progressively increase relative to the propionic acid residue.

One other factor may also contribute in part to the greater effectiveness of the odd-carbon fatty acids of longer chain-length. It was observed, for the whole series of fatty acids, that, as the chain-length increased, progressively smaller concentrations were required to produce maximal amplitude effects. An explanation for this finding may reside in the greater affinity of the longer-chain fatty acids for the β-oxidation enzyme surface, a concept which derives support from the increase in the adsorption coefficients of fatty acids at surfaces, as chain-lengths increase. With respect to the odd-carbon series, such an affinity relationship would act to the advantage of the longer-chain members in their competition with propionic residues.

No information is at present available as to the usefulness of higher fatty acids, such as oleic, palmitic, and stearic, as substrates for smooth muscle contractility, because of the extremely low solubility of their sodium salts in Krebs solution. They were ineffective in dilute suspensions.‡

Summary. A number of substances have been studied with respect to their influence on the contraction of excised segments of rabbit small intestine. The results have been discussed in relation to present concepts of intermediary metabolism. Particular attention has been given to fatty acids, from propionic through pelargonic. With the exception of propionic, all appear to provide energy for smooth muscle contraction; and the characteristics of their behaviour were compatible with the concept of β-oxidation.

‡ Since this manuscript was submitted, oleic acid has been found to partially restore contraction amplitude. It was introduced as a sodium oleate sol (100 mg %) in water along with enough NaCl to maintain isotonicity. The effective concentration of oleic acid could not be determined because of its partial precipitation.

We wish to acknowledge the technical assistance of Mathilda Fischl, Alice Kantor, and Rita Baffa.

15301

Experimental Sinoauricular Block.*

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Sinoauricular block is a rare disturbance either of stimulus formation in the sinus node or of transmission of the stimulus to the auricle. In analogy with tracings obtained in auriculoventricular conduction disturbances, sinoauricular block is diagnosed whenever, with a regular sinus mechanism, pauses occur without evidence of auricular activity. These pauses are more or less approximate multiples of a normal period.

A disturbance of stimulus formation in the sinus node or of stimulus conduction in the sinoauricular junctional fibers is understandable during vagus stimulation or carotid pressure; the occurrence of sinoauricular block

* The expenses of this investigation were defrayed by a grant from the United Hospitals Fund.

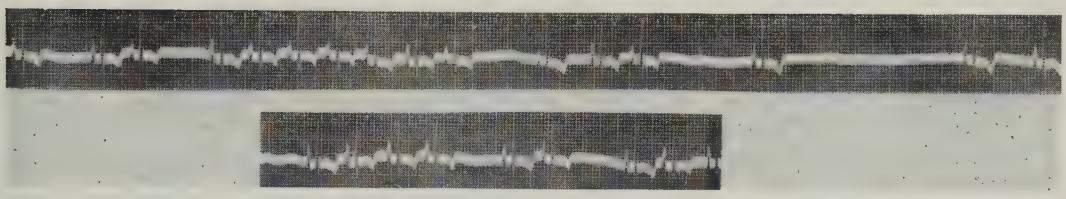

Fig. 1a and b. Sinoauricular block following subepicardial injection of strophosid at the lower end of the sinus node.

in patients with arteriosclerosis of the sinus node arteries is also to be expected. It is difficult to explain, however, the sinoauricular block which follows the administration of digitalis or the form which is occasionally seen in patients with myocardial involvement. The sinus node represents a relatively large structure with a length of more than 25 mm, it is united with the common auricular fibers by innumerable junctional fibers which spread in all directions. Furthermore, one must assume that all specific fibers of the sinus node are able to form automatic stimuli. It is hard to conceive how any but a very extensive process could lead to a sinoauricular block; the simultaneous periodical block of the sinoauricular conduction in all junctional fibers due to digitalis action seems impossible.

Therefore for many years Lewis spoke only of "sinoauricular block, so-called"[1] and Clerc discusses "le blocage sinoauriculaire ou supposè tel."[2]

In complete accord with these facts was the experience that in order to obtain sinoauricular block experimentally, it is necessary to isolate the sinus node on all 4 sides[3] to clamp the sinus node artery and thus damage the tissue of the whole node,[4] or to inject pituitary extracts which cause coronary spasm.[5] All experimentators who have tried to produce a sinoauricular block agree that this disturbance cannot be easily created.

In experiments which were performed for other purposes we registered electrocardiograms showing sinoauricular block. The method by which these tracings were obtained seems worth reporting because it may serve to explain some of the clinical cases of sinoauricular block.

Experimental Results. In experiments in which extrasystoles were induced by the local application of digitalis or strophanthin to the sinus node area,[6] the subepicardial injection of these drugs led in some instances to immediate cardiac standstill and to temporary arrhythmias. These disturbances appeared so soon after the application that it seemed unlikely that they are due to a specific effect of the injected drugs. It was actually found that hypertonic solutions of barium, sodium, or calcium chloride evoked the same effect when injected.

Dogs were used in all experiments. During anesthesia with morphine and nembutal the heart was exposed while artificial respiration was started. The subepicardial injection was done with a fine needle, attached to a tuberculin syringe. The amount injected never exceeded 0.1 cc. The epicardium always became detached at the site of injection and was visible as a grey covering over a small vesicle. The arrhythmias could be initiated from all areas of the sinus node but they appeared most often if the injection was made at the lower end of the node. In some experiments the same disturbances of rhythm were also obtained when the injection was made a few millimeters beyond the sinus node area toward the appendix of the right auricle.

Fig. 1a was obtained in an experiment in which strophosid was injected at the lower end of the sinus node. A regular sinus rhythm existed with a rate of 127-136, that is, the single cycles measured between 0.44

1 Lewis, T., *The mechanism and graphic registrations of the heart beat*, London, 1925.

2 Clerc, A., *Les arythmies en clinique*, Paris, 1925.

3 Cohn, A. E., Kessel, L., and Mason, H. H., *Heart*, 1911-12, **3**, 311.

4 Scherf, D., *Z. f. d. ges. exp. Med.*, 1927, **57**, 188.

5 Resnik, W. H., *Arch. Int. Med.*, 1925, **36**, 788.

6 Scherf, D., *Exp. Med. and Surg.*, 1944, **2**, 70.

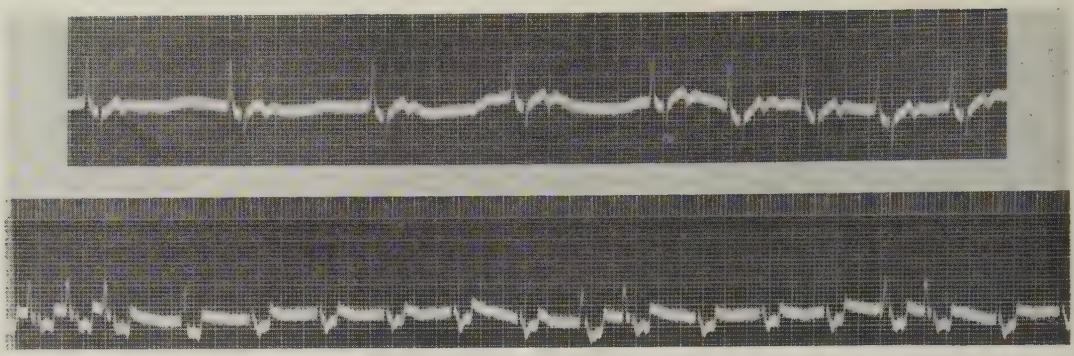

Fig. 2 (top). 2:1 sinoauricular block after injection of a 2% solution of sodium chloride over lower end of sinus node.

Fig. 3 (bottom). Sinus block and a-v rhythm after injection of 10% solution of sodium chloride over the middle portion of the sinus node.

and 0.47 second. Immediately following the injection, pauses occurred in the otherwise regular rhythm. Fig. 1a was registered about 10 seconds later. After the first and the third beats in Fig. 1a pauses appear which measure 0.90 second, that is, they are equal to 2 x 0.45 second. The third pause in the tracing is 1.06 seconds long but it is terminated by an auricular beat with a different P-wave, originating presumably in a different focus. The fourth pause in the tracing is 1.40 seconds long (3 x 0.47 second), and the last one measures 2.42 seconds (5 x 0.48 second). About 2 minutes later, with sinus periods of 0.44 to 0.48 second (Fig. 1b) the pauses were 0.88 and 0.96 second long, exactly twice the length of a normal period. From the length of these pauses and from their comparison with the other sinus periods, the conclusion is justified that the tracings exhibit sinoauricular block.

In some of the other experiments a marked bradycardia appeared following the injection and the rate then suddenly doubled. Thus the tracing of Fig. 2 was recorded after the injection of 0.1 cc of a 2% solution of sodium chloride into the lower region of the sinus node, the P-waves in this experiment were unusually tall and they are therefore followed by pronounced Ta-waves. The length of the first 4 long periods measures 1.26, 1.25, 1.24, and 1.23 seconds. The following short periods measure 0.67, 0.66, 0.66, and 0.64 second. It is well known from clinical and from experimental sinoauricular block that the long period caused by the block is usually shorter than 2 normal periods.

The effect of the subepicardial injection of a 10% solution of sodium chloride over the middle of the sinus node is visible in Fig. 3. The tracing was obtained a few seconds after the injection. The length of the first 2 periods at the beginning of the tracing measures 0.36 and 0.34 second. The period following has a length of 0.72 second (2 x 0.36 second). Then for 3.60 seconds (10 x 0.36 second) no P-waves are visible and atrioventricular rhythm prevails. Following this long cessation of the normal sinus mechanism a sinus period of 0.38 second can be measured. The next long pause measures 2.34 seconds (6 x 0.39 second) and the last short one is 0.38 second. After the disappearance of the sinus block, injection with Digilanid C was repeated over the same area and the same result was obtained.

In the majority of cases the disturbance of the activity of the sinus node appeared so quickly that the end of the first (and usually longest) standstill could just be recorded after the injection was completed. In other experiments the arrhythmia appeared after about one minute, and only exceptionally a little later. The disturbance never lasted longer than 6 minutes.

In view of the appearance in an otherwise regular rhythm of pauses having a length which is a multiple of a normal period and during which evidence of auricular activity is missing, sinoauricular block may be assumed to exist in these tracings. In many tracings there was no standstill of the heart

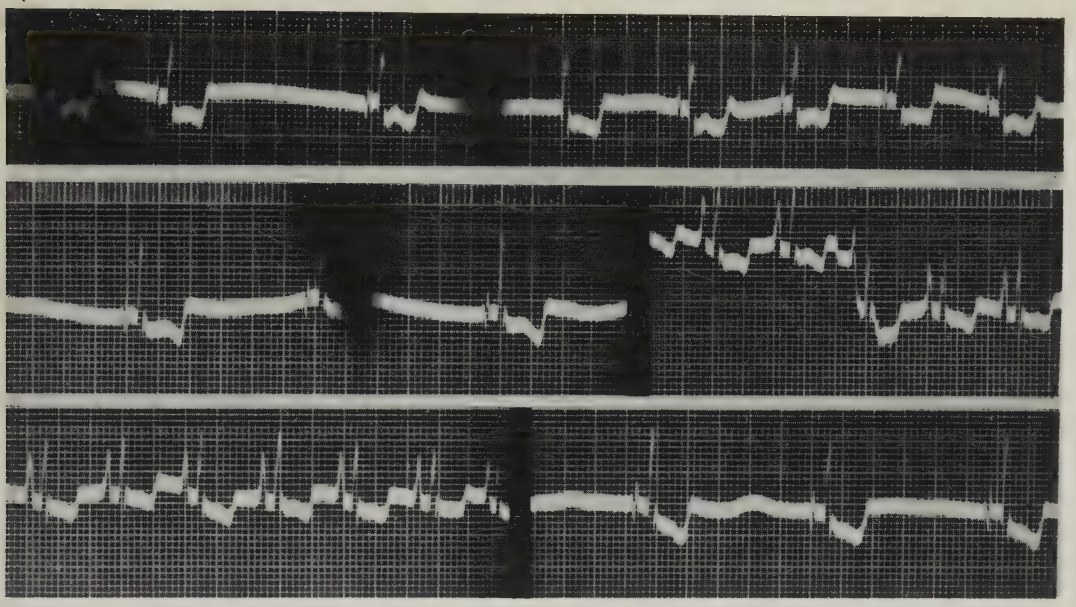

Fig. 4a, b and c. Following repeated injection of a 2% solution of sodium chloride over different portions of the sinus node the same type of sinoauricular block appears; severing of both vagi does not change the result.

as in Fig. 1 and 2, but an atrioventricular nodal rhythm appeared as in Fig. 3. In some of these cases the inverted P-waves preceded the ventricular complexes at a normal distance (coronary sinus rhythm); in other cases P-waves were not visible and were probably buried in the QRS-complexes; in rare cases they appeared even after the QRS-complex but before the T-wave (lower auriculoventricular rhythm). Interference between sinus and auriculoventricular rhythm was also seen.

Whenever the sinus node centers are inhibited, the "secondary" centers in the auriculoventricular node perform the duty of pacemaker of the heart. The automaticity of these centers is only slightly less developed than that of the sinus node centers and often it is equally high. Therefore it has been repeatedly pointed out that in order to obtain tracings with bradycardia or long standstill as in Fig. 1 and 2, not only is a damage of the sinus node necessary, but also an additional depression of the centers of the auriculoventricular node.[1,7]

In 47 experiments only 9 instances of sinoauricular block like Fig. 1 and 2 were observed. In 14 other instances only deeper centers of the auriculoventricular node became active as in Fig. 3. In several experiments repitition of the same injection or injection of other substances produced the same results as the first injection.

Severing of both vagi in the neck did not influence the results.

In the experiment in which Fig. 4 was obtained, subepicardial injection of a 2% solution of sodium chloride over the head of the sinus node led to a change of rhythm. The first beat in Fig. 4a is an automatic "escape beat." The second is preceded by a P-wave of abnormal appearance and is probably conducted from some lower area of the sinus node. After 1.33 seconds (2 x 0.66 second) another sinus beat appears. The following sinus beat comes after a pause of 1.95 seconds (3 x 0.65 second). This long pause is also shortened by an escaped beat from a deeper center. The length of the 3 following sinus periods measures 0.66, 0.65, and 0.65 second. The longer pauses therefore closely approximate double and triple the length of one normal period.

Fig. 4 was obtained from the same animal

[7] Eyster, J. A. E., and Meek, W. J., *Arch. Int. Med.*, 1917, **19**, 117.

about 20 minutes later. Again a bradycardia with a period of 1.36 seconds (2 x 0.68 second) appeared after injection of 0.1 cc of a 2% solution of sodium chloride at the lower end of the sinus node. A few minutes later both vagi were severed in the neck and a regular sinus rhythm was obtained with large positive P-waves and a rate of 125 beats per minute with each period measuring 0.48 second. The injection was then repeated at the same area and the same tracing as before was obtained. A bradycardia appeared (Fig. 4c) with P-waves of the same abnormal appearance as those following the previous injections, and the length of the period is again 1.36 seconds. Between Fig. 4b and Fig. 4c, 9 QRS-complexes were cut out.

Discussion. The tracings described in the preceding section show that focal mechanical stimulation of the tissue in the neighborhood of the sinus node may lead to the appearance of sinoauricular block. It is difficult to assume any other than a reflex mechanism on the sinus node tissue for this disturbance. A reflex vascular spasm of the arteries supplying the sinus node would not lead to the immediate appearance of the changes of rhythm described above; it would not cause their appearance for from one to 3 minutes. It was impossible to decide whether the inhibition concerns stimulus formation or conduction of the stimulus from the sinus node to the auricle.

The same reflex inhibition which leads to sinoauricular block in some instances also depressed the automaticity in the auriculoventricular centers so that long cardiac standstill resulted.

It is possible that focal lesions in the sinus node or in the surrounding tissue, for instance an inflammation as in the case of Hume,[8] cause sinoauricular block by a similar reflex mechanism. The same mechanism may explain cases of rheumatic fever and sinus block. Here also, focal inflammatory lesions frequently occur. Otherwise sinoauricular block could be expected only if the lesion destroyed all but a small area of the sinus node tissue, and all but one of the junctional bridges between the sinus node and the surrounding auricular muscle.

The instances of sinoauricular block following digitalis medication, comprising approximately 50% of the known cases,[9,10] can be explained only with difficulty. The occurrence of focal myocardial lesions following huge doses of digitalis is known, but these lesions have never been seen with certainty in man; even if they do occur, they can be expected only after much larger doses than those which usually suffice to elicit sinoauricular block.

Summary. The subepicardial injection of 0.1 cc of digitalis or of a strophanthin preparation, as well as of other substances, over the sinus area leads to the immediate but transient appearance of sinoauricular block. This phenomenon is attributed to a reflex inhibition of the sinus node centers caused by the mechanical stimulus and not to a specific effect of the injected substances. In some instances the centers of the auriculoventricular node are also inhibited.

[8] Hume, W. E., *Heart*, 1913-14, **5**, 25.

[9] Barlow, P., *Lancet*, 1927, **1**, 65.

[10] Levine, S. A., *Arch. Int. Med.*, 1916, **17**, 153.

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Respiration in *Macaca mulatta* (Rhesus Monkey).

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During the course of an investigation of the effects of phosgene on the respiratory exchange of normal animals, it was found that the available literature contains no information on the respiratory exchange in the restrained, unanesthetized, non-fasting, normal *Macaca mulatta* (Rhesus monkey) despite the extensive use of this species in laboratory investigations. To facilitate the evaluation of metabolic, inhalation, and other studies in monkeys and to permit comparison with the data for other species, the average respiratory rate, tidal air, and minute volume were measured in 39 unanesthetized, untrained, non-fasted macaques. Tidal air and minute volume were calculated in terms of both body weight and body surface area, in an effort to determine which of the latter provides the better basis for expressing respiratory data in this species.

Several months after the original investigation had been completed, it was felt that it would be interesting to include with the other data the respiratory exchange, metabolic rate, and coefficient of oxygen utilization in non-basal monkeys. Such determinations were made on the 4 animals remaining in the laboratory colony.

Methods. Immediately before the experiment, the unanesthetized, untrained monkey was weighed and restrained in a spread-eagled, supine position on an animal board. Then, to reduce external stimuli, the eyes and ears were carefully bandaged, and the board was raised to the vertical plane to approximate more closely the semi-erect, normal, simian posture. By means of a face-piece, formed with elastic, adhesive bandage, and low resistance respiratory valves,[1,2] the exhaled air was directed into a 4.5 liter McKesson Metabolor, giving a continuous record of time, volumes respired, and individual expirations. The carbon dioxide absorbent and the filters were removed from the spirometer to prevent the absorption of carbon dioxide from the exhaled air and to reduce the resistance of the system. Neither the method for restraining the animals nor the facepiece-valve-spirometer system offered any interference to respiration.

On the basis of dentition and body weight,[3] the age of the monkeys was estimated to range from 14 to 36 months. All were sacrificed subsequently, and autopsies were performed. Only normal animals are reported in this series. A diet of fruits, purina fox chow, and water was given *ad libitum* prior to the experiment. No sedation of any kind was administered except as noted below. The approximate mean room temperature and relative humidity were 76°F and 47.6%, respectively.

To give an indication of the state of excitement of the animals, pulse rates were determined within 60 seconds after the completion of each respiratory record. During the period of observation, which was from 1.0 to 2.8 minutes, the animals were relatively quiescent. Whenever an occasional animal did struggle, the records were discarded and the experiment repeated.

The respiratory and metabolic data on the 4 macaques were obtained by use of an oxygen-filled, calibrated 300 cc spirometer with an absorber for the exhaled CO_2. Oxygen consumption was measured in the usual manner for indirect calorimetry. Inasmuch as at the later date satisfactory elastic, adhesive bandage was not available for the

* Capt., CWS, AUS.

† Capt., MC, AUS.

1 Tobias, J. M., and Weston, R. E., in press.

2 Weston, R. E., and Karel, L., in press.

3 Hartman, C. G., and Straus, W. L., Jr., *The Anatomy of the Rhesus Monkey*, Williams and Wilkens Co., Baltimore, 1933.

TABLE I.
Summary of Respiratory Data for *Macaca mulatta*.

Sex	Wt. kg	Surface m^2	Resp. per min	Tidal air			Minute volume			Pulse per min
				cc	cc/kg	cc/m^2	cc	cc/kg	cc/m^2	
M	1.99	.185	37	19.4	9.7	104.8	714	359	3790	187
M	2.02	.187	29	24.6	12.2	131.2	700	347	3740	208
M	2.03	.187	45	19.8	9.8	106.0	886	436	4730	188
M	2.07	.190	35	19.0	9.2	100.5	661	319	3512	214
M	2.19	.198	34	15.9	7.3	80.6	544	249	2754	210
M	2.19	.198	45	21.3	9.7	107.5	948	433	4791	240
F	2.19	.198	31	17.7	8.1	89.4	554	253	2805	228
F	2.21	.199	41	17.5	7.8	88.2	716	324	3601	164
F	2.24	.200	47	19.2	8.6	96.2	901	402	4503	230
F	2.32	.205	37	21.6	9.3	105.6	796	343	3929	246
M	2.35	.207	36	26.2	11.2	126.7	931	396	4898	218
M	2.53	.218	35	31.0	12.2	142.3	1075	425	4935	198
F	2.53	.218	43	24.9	9.9	114.7	1069	422	4910	216
F	2.54	.218	40	22.7	8.9	104.6	908	358	4173	190
F	2.55	.219	34	22.0	8.6	100.7	752	295	3440	224
F	2.56	.219	35	16.3	6.4	74.7	572	223	2612	214
F	2.58	.220	38	33.2	12.9	150.9	1277	495	5806	212
F	2.58	.220	33	28.7	11.1	130.4	937	363	4258	216
F	2.58	.220	41	32.7	12.7	148.9	1328	515	6036	230
F	2.61	.222	38	21.3	8.2	96.0	800	307	3600	204
F	2.62	.222	27	15.0	5.7	67.5	405	155	1835	156
M	2.64	.223	37	34.7	13.2	155.5	1267	480	5668	234
M	2.67	.225	44	28.2	10.6	125.2	1244	466	5520	246
F	2.72	.228	29	52.4	19.3	229.9	1505	554	5600	254
F	2.73	.228	24	29.2	10.7	127.8	693	254	3078	164
M	2.75	.230	49	31.2	11.4	135.9	1530	556	6660	234
M	2.75	.230	36	32.0	8.4	139.4	1164	424	5062	232
M	2.78	.232	43	41.2	14.8	177.7	1768	636	7630	150
M	2.81	.233	20	27.7	9.9	119.0	554	198	2381	250
M	2.84	.235	43	38.4	13.5	163.9	1670	588	7113	240
M	2.87	.236	41	28.2	9.8	119.6	1152	401	4871	274
M	2.89	.237	44	39.5	13.7	166.5	1734	600	7300	—
F	2.95	.241	33	18.6	6.3	77.2	618	210	2571	216
F	3.10	.249	43	15.8	5.1	63.6	678	219	2730	228
M	3.10	.249	38	26.4	8.5	106.2	1008	325	4055	226
F	3.18	.253	48	41.7	13.1	164.9	2004	630	7923	226
M	3.35	.262	32	16.3	4.9	62.3	522	156	2000	214
F	3.54	.271	37	35.0	9.9	129.1	1288	364	4742	—
M	3.55	.272	46	36.5	10.3	134.4	1686	475	6193	190
Mean (m)	2.63	.223	37.6	26.8	10.1	119.6	1014	383	4532	215
Std. Dev. (σ)	0.41	.022	6.6	8.7	2.8	33.4	403	129	1554	27
Std. Error (ϵ)	0.07	.004	1.1	1.4	0.5	5.3	64	21	248.8	4.5
Coef. of Var. (σ/m) in %	15.6	10.1	17.4	32.5	28.2	28.0	39.7	33.7	34.3	12.7

facepiece, which must form an absolute seal around the nose to obviate leakage, the monkeys were tracheotomized. Determinations on these animals were made both prior to and subsequent to nembutalization. The tracheotomies were performed under local anesthesia, 1.0 cc of a 2% procaine (with adrenalin) solution being used. The distal end of the glass, tracheal cannula consisted of a male half of a standard taper joint by means of which the animal was connected to the apparatus.[1,2] Nembutalization was achieved by the intraperitoneal injection of 10 mg per kg of nembutal 20 minutes prior to the metabolism tests.

Results. The average values for minute volume, respiratory rate, and tidal air were determined for each animal from the spirom-

TABLE II.
Respiratory Data on Monkeys Before and During Exposure to a Phosgene Concentration of 1.38 mg per Liter.

Exper. conditions	Respiratory rate per min			Average tidal air			Minute volume		
	Mean	σ	ϵ	Mean	σ	ϵ	Mean	σ	ϵ
	cc	cc	cc	cc/kg	cc/kg	cc/kg	cc/kg	cc/kg	cc/kg
Normal respiration	37.6	6.6	1.1	10.1	2.8	0.5	383	129	21
Respiration during phosgene exposure	29.0	10.7	1.8	6.8	1.9	0.3	188	72.5	11.9

eter records. These data are summarized in Tables I and III.

Body surface areas in square centimeters were calculated from the formula:

$$S = KW^{2/3}$$

where W is the weight in grams and K is a constant which, for monkeys, equals 11.7 with an average deviation of $\pm$ 5.4%.[4] The arithmetic means, standard deviations, standard errors, and coefficients of variation were determined in the usual manner.[5]

Under the conditions of these experiments, young adult *Macaca mulatta,* weighing 2.63 kg on the average, had a respiratory rate of 37.6 per minute (coefficient of variation = 17.4%), a mean tidal air of 26.8 cc (c. v. = 32.5%), and a mean minute volume of 1014 cc (c. v. = 39.7%). Expressed in terms of body weight or body surface area, the mean tidal air was 10.1 cc per kilogram (c. v. = 28.2%) or 119.6 cc per square meter (c. v. = 28.0%), and the mean minute volume was 383 cc per kilo (c. v. = 33.7%) or 4532 cc per square meter (c. v. = 34.3%).

Because the calculation in terms of body surface area yielded no smaller coefficient of variation than did the simple expression in terms of body weight, minute volumes were calculated in terms of another unit, the body weight in kilograms raised to the 0.73 power, which has been recommended as the basis of reference for metabolic and other data.[6] However, this calculation resulted in the following expression for the minute volume:

$$\text{Minute volume in cc} = 499W^{0.73},$$

(standard deviation (σ) = $\pm$ 173.2, standard error (ϵ) = $\pm$ 27.7) with a coefficient of variation of 34.7%, which is no smaller than that obtained from the calculations in terms of either the surface area or the body weight to the first power.

The average pulse observed in these unanesthetized, untrained monkeys was 215 per minute (c. v. = 12.7%), a value which is apparently too high for the basal state. With the exception of the report by Hartman *et al.*[7] on fetal and maternal heart rates, there are no data on the pulse rates of unanesthetized, untrained, normal macaques in the available literature for comparison. The rates reported by Hartman *et al.*,[7] however, agree well with those found for normal nongravid macaques in the present report. Although it was hoped that the pulse rate would serve as an index of the degree of excitability of the individual animals, no distinct correlation could be made between pulse rate and any of the respiratory data.

A comparison of the data obtained on the normal monkeys prior to and during their exposure to phosgene (Table II) indicates the extent of inhibition of the respiratory exchange during contact with the noxious vapors.[2] Whereas the uninhibited monkeys had a mean respiratory rate, tidal air, and minute volume of, respectively, 37.6 per minute, 10.1 cc/kg, and 383 cc/kg, the corresponding values during phosgene exposure

[4] Lee, M. O., and Fox, E. L., *Am. J. Physiol.,* 1933, **106**, 91.

[5] Fisher, R. A., *Statistical Methods for Research Workers, 8th Ed.,* G. E. Stechert and Co., New York, 1941.

[6] Benedict, F. G., *Vital Energetics, Carnegie Inst. Wash. Publ. No. 503,* Wash., 1938.

[7] Hartman, C. G., Squier, R. R., and Tinklepaugh, O. L., PROC. SOC. EXP. BIOL. AND MED., 1930-31, **28**, 285.

TABLE III.
Respiratory Exchange, Metabolic Rate and Coefficient of Oxygen Utilization in 4 Tracheotomized, Non-basal *Macaca mulatta* Before and After Intraperitoneal Administration of 10 mg of Nembutal/kg.

No.	Wt.	Sex	Pulse before nemb.	Tidal air		Resp./min.		Min. vol.		Cals/kg/24 hrs		Cals/sq.m/24 hrs		Coef. of O_2 util	
				Before	After	Before	After	Before	After	Before	After	Before	After	Before	After
				cc/kg	cc/kg			cc/kg	cc/kg					%	%
1	3.26	F	222	15.9	14.5	31	35	493.1	508.0	99.52	90.67	1262.44	1150.07	3.22	2.85
2	3.52	M	240	12.5	11.4	43	39	538.8	442.9	79.11	90.55	1027.61	1176.12	2.35	3.27
3	3.56	M	260	21.0	21.7	48	46	1006.1	998.5	93.90	116.25	1224.47	1515.89	1.53	1.91
4	3.61	F	228	19.8	18.5	50	42	988.2	774.9	70.50	73.61	1034.62	966.24	1.31	1.56
Mean	3.49			17.3	16.5	43	41	756.6	681.1	85.76	92.77	1137.29	1202.08	2.10	2.40

were 29.8 per minute, 6.8 cc/kg, and 188 cc/kg. Statistically, these differences are highly significant.

The complete protocols for the experiment on the tracheotomized macaques appear in Table III. For the non-nembutalized monkeys, the mean heat production was 85.76 Cals/kg/24 hrs or 1137 Cals/sq.M/24 hrs, which is approximately 180% of the basal values reported by Benedict[6] and Rakieten.[8] Twenty minutes after 10 mg/kg intraperitoneal doses of nembutal, the mean heat production was 92.77 Cals/kg/24 hrs or 1202.08 Cals/sq.M/24 hrs. Previously, Rakieten[8] had reported a similar increase in one rhesus monkey after amytal.

The mean coefficient of oxygen utilization was 2.10% for the non-nembutalized and 2.40% for the "sedated" monkeys. In Table IV a further comparison is made between values calculated from Benedict,[6] Rakieten's[8] data, and the data on the 4 tracheotomized macaques.

Discussion. On the basis of studies on the basal metabolic rate, Kleiber[9] proposed that the minute volume (or tidal air per minute as it is termed in his report) for any resting, fasting laboratory animal may be determined from the formula:

$$\text{Minute volume in cc} = 212W^{3/4}$$

where W is the body weight in kilograms. He gave no indication of the statistical range of the values calculated from this formula but, as evidence supporting its application, cited the almost exact identity of the calculated minute volume with that observed in one deeply anesthetized, tracheotomized, 3.5 kg Rhesus monkey in which the observed average minute volume was 157 cc per kilogram. The significance of data obtained on one animal is questionable. Moreover, as Kleiber noted, the animal studied was deeply narcotized, a circumstance which depresses not only the metabolic rate but also the respiratory center.

Actual measurement of minute volumes in 39 unanesthetized, non-fasted, untrained Rhesus monkeys (Table I) revealed much higher

[8] Rakieten, N., *J. Nutrition*, 1935, **10**, 357.

[9] Personnel of U. S. Navy Med. Res., Unit No. 1, and Kleiber, M., *Science*, 1944, **99**, 542.

TABLE IV.
Comparison of Basal and Non-basal Heat Production of *Macaca mulatta*.

Benedict* (basal)		Rakieten† (basal)			Karel and Weston‡ (Tracheotomized, non-basal)		
Wt.	Cals/kg/24 hrs	Wt.	Sex	Cals/kg/24 hrs	Wt.	Sex	Cals/kg/24 hrs
kg		kg			kg		
3.24	52.2	2.7	M	50.5	3.52	M	79.11
3.56	55.9	2.8	M	52.2	3.56	M	93.90
3.84	44.8	2.9	M	48.9	3.26	F	99.52
3.97	51.9	2.9	M	51.0	3.61	F	70.50
4.04	48.0	3.1	M	43.6			
4.18	47.1	3.1	F	49.1			
4.24	46.7	3.5	F	45.1			
4.34	45.4	3.5	F	54.4			
4.35	50.1	3.6	F	41.3			
4.42	49.1	3.6	F	48.5			
4.50	46.7	3.7	F	48.7			
4.58	38.9						
4.73	51.2						
5.10	44.3						
Mean (basal)	48.0	Mean (basal)		48.5	Mean (non-basal)		85.76

* Values calculated from plotted points on graph made by Dr. George L. Streeter, Dept. of Embryology, The Johns Hopkins University, Baltimore, Md., on "basal" *Macaca mulatta*. A few of the points represent averages which include experiments made both with and without the use of nembutal.[6]

† Data from Table I.[8]

‡ From Table III.

values than would be expected from Kleiber's formula. Consequently, calculating the minute volume in terms of the basis of reference suggested by the Committee on Animal Nutrition of the National Research Council[6] for computing metabolism yielded a constant which is 2.35 times as great as the constant suggested by Kleiber, with a wide statistical range. The 39 untrained monkeys in this series were not observed under basal conditions as indicated by the pulse rates, but the unanesthetized macaque, as others have noted,[6] under the usual laboratory conditions is seldom basal, particularly if subjected to any experimental procedures.

The great variability of the respiratory exchange in unanesthetized macaques is another example of individual variation within a presumably uniform population. The animals in this series did not differ markedly in age, weight, nutritional state, or body surface area, and the conditions of the experiment were well controlled. Nevertheless, the large coefficients of variation reported in this paper indicate the wide deviations from the mean in the respiratory response of the individual animals.

Since the respiratory exchange is a reflection of the metabolic activity of the organism, which, in turn, is a function of the body mass, calculation of the tidal air and minute volume in terms of body weight did decrease the coefficient of variation somewhat, as would be expected. That recalculating these data in terms of body surface area or of another exponential function of the body weight, the 0.73 power, did not result in an additional decrease in the coefficients of variation is not surprising. For, as Benedict[6] has emphasized, it is futile to attempt by mathematical means to achieve a uniform expression of even basal metabolic findings despite the "great weight given to complicated mathematical treatment by various writers and the acceptance of these formulas by less critical authors." When to the differences in individual metabolic rates there are added the many other factors which might produce changes in the respiration of any single laboratory animal, the observed range of individual values is to be expected.

It will be noted that the respiratory exchange, particularly in animals No. 3 and 4, of the tracheotomized series was appreciably greater than that found in the series of 39

monkeys. Since, however, the former group was tracheotomized and immediately prior to the tracheotomy had been injected with procaine plus adrenalin, this may account for the substantial difference.

While no positive inferences drawn from the data obtained on the few tracheotomized macaques should be applied to non-tracheotomized animals, it is provocative to note that by application of the coefficient of oxygen utilization (2.10%) to the basal values reported by Benedict[6] and by Rakieten,[8] one arrives at a mean ventilation rate which is almost the same as the value of 383 cc per kg per minute found as the mean minute volume in the present series of 39 monkeys.

The low coefficient of oxygen utilization is probably the result of the increased minute volume. Benedict and Benedict[10] have reported appreciably increased ventilation rates and decreased oxygen absorption in normal subjects doing mental multiplications. The coefficient of oxygen utilization calculated from the data of Benedict and Benedict[10] fell as low as 2.6%.

Summary. 1. The average respiratory rate, tidal air, and minute volume were measured in 39 unanesthetized, untrained, young monkeys *(Macaca mulatta)* with an average weight of 2.63 kg ($\sigma = \pm 0.41$ kg, $\epsilon = \pm 0.07$ kg).

2. Expressing the tidal air or minute volume in terms of body weight in kilograms, body surface area in square meters, or body weight in kilograms raised to the 0.73 power resulted in practically identical coefficients of variation.

3. The mean values observed were as follows:

a. Respiratory rate: 37 per minute ($\sigma = \pm 6.6$; $\epsilon = \pm 1.1$).

b. Tidal air: 10.1 cc per kg ($\sigma = \pm 2.8$ cc; $\epsilon = \pm 0.5$ cc) or 119.6 cc per square meter ($\sigma = \pm 33.4$ cc; $\epsilon = \pm 5.3$ cc).

c. Minute Volume: 383 cc per kg ($\sigma = \pm 129$ cc; $\epsilon = \pm 21$ cc) or 4532 cc per square meter ($\sigma = \pm 1554$ cc; $\epsilon = \pm 248.8$ cc) or 498.7 cc per kg body weight raised to the 0.73 power ($\sigma = \pm 173.2$ cc; $\epsilon = \pm 27.7$ cc).

4. No correlation was found between the animals' pulse rate (mean = 215 per minute) ($\sigma = \pm 27$; $\epsilon = \pm 4.5$), which was intended as an index of excitability, and any of the respiratory data.

5. The respiratory rate, tidal air, and minute volume were significantly inhibited in monkeys exposed to a phosgene concentration of 1.38 mg/1.

6. a. The mean heat production determined by the indirect method in 4 unanesthetized, untrained, tracheotomized macaques with an average weight of 3.49 kg was 85.76 Cals/kg/24 hrs and 1137.29 Cals/sq.M/24 hrs—approximately 180% of the basal values reported in the literature.

b. The mean heat production of the same macaques sedated with 10 mg/kg of nembutal intraperitoneally was 92.77 Cals/kg/24 hrs and 1202.08 Cals/sq.M/24 hrs.

7. The average coefficient of oxygen utilization in these 4 monkeys was 2.10% for the non-sedated and 2.40% for the nembutalized macaques.

The authors wish to thank Lt. Donald R. LaGrave for technical assistance in conducting these experiments.

[10] Benedict, F. G., and Benedict, C. G., *Carnegie Inst. Wash.* (Nutrition Laboratory), 1933.

15303

Relative Growth of the Kidney in Male Rats.

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The weights of organs and of body components are usually recorded as percentage of body weight or in some other simple arithmetic ratio to body weight. The cumbersome quality of such presentation is apparent especially when body weight varies over a wide range. The percentage value generally changes during growth but may become identical for widely different body weights. Klatt[1] calling attention to this points out the very advantageous general applicability of the power formula $y = ax^b$ in which y = the body part or organ; x = body weight and a and b are constants. This had been the basis for the work of DuBois and of Lapicque which successfully solved a number of problems concerned with the relative weight of the brain. He also points out that with an entirely different approach the same formula had been applied to surface and energy metabolism. Moulton[2] who dealt with the interrelation of body weight, body surface, body nitrogen, weight of blood etc., showed that the handling of such data and the evaluation of the 2 constants can without lengthy calculation be carried out very simply and with sufficient accuracy by graphical methods.

Since then the power formula has found wide application in the hands of Huxley,[3] Needham,[4] Brody,[5] Kleiber and associates,[6] Adolph[7] and others in various problems concerned with organ weights, weights of chemical components of the body, linear measurements of body parts, and measures of physiological functions.

This report presents individual kidney weights and body weights of 150 male stock diet rats and mean weights of 15 consecutive groups of 10 animals each. Kidney weight is given as the combined weight of the 2 kidneys.

In Fig. 1 the small circles are individual weights and the crosses represent the mean values for groups of 10. Both scales are logarithmic and $y = ax^b$ plots a straight line. It is seen that the relative growth of the kidney may be divided into 3 phases, each represented by a straight line whose slope indicates the relative rate of growth of kidney and body. As shown in Table I the ratio of kidney weight to body weight (expressed as %) definitely increases during the first phase; it approximates constancy during the second phase; during the third phase it definitely decreases. The percentage values of the first phase repeat themselves in the third phase in reverse order. The same in-

TABLE I.
Body and Kidney Weight Data Averaged in Groups of 10.

Mean age days	Mean body wt. g	Mean wt. of 2 kidneys mg	%
0.1	5.45	44.3	.812
1.8	6.70	61.8	.922
4.8	9.08*	96.6	1.063
6.2	11.75	122.4	1.041
10.1	17.04	176.7	1.036
12.3	23.75	244.3	1.028
17.4	35.72	397.1	1.111
27.0	49.54*	555.1	1.120
33.2	70.08	744.0	1.061
42.7	110.1	1060	.963
63.6	162.9	1402	.860
79.1	195.7	1694	.865
89.4	250.4	1944	.776
157	303.4	2379	.784
281	408.7	2867	.701

* Body weights at which the slope changes of Fig. 1 occur.

[1] Klatt, B., *Biol. Zentr.*, 1919, **39**, 406.

[2] Moulton, C. R., *J. Biol. Chem.*, 1916, **24**, 299.

[3] Huxley, J. S., *Problems in Relative Growth*, London, 1932.

[4] Needham, J., *Biochemistry and Morphogenesis*, Cambridge, 1942, p. 532 ff.

[5] Brody, S., Davis, H. P., and Ragsdale, A. C., *Mo. Agr. Exp. Sta. Res. Bull.*, 1937, No. 262.

[6] Weymouth, F. W., Field, J., and Kleiber, M., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 367.

[7] Adolph, E. A., *Physiological Regulations*, Lancaster, 1943.

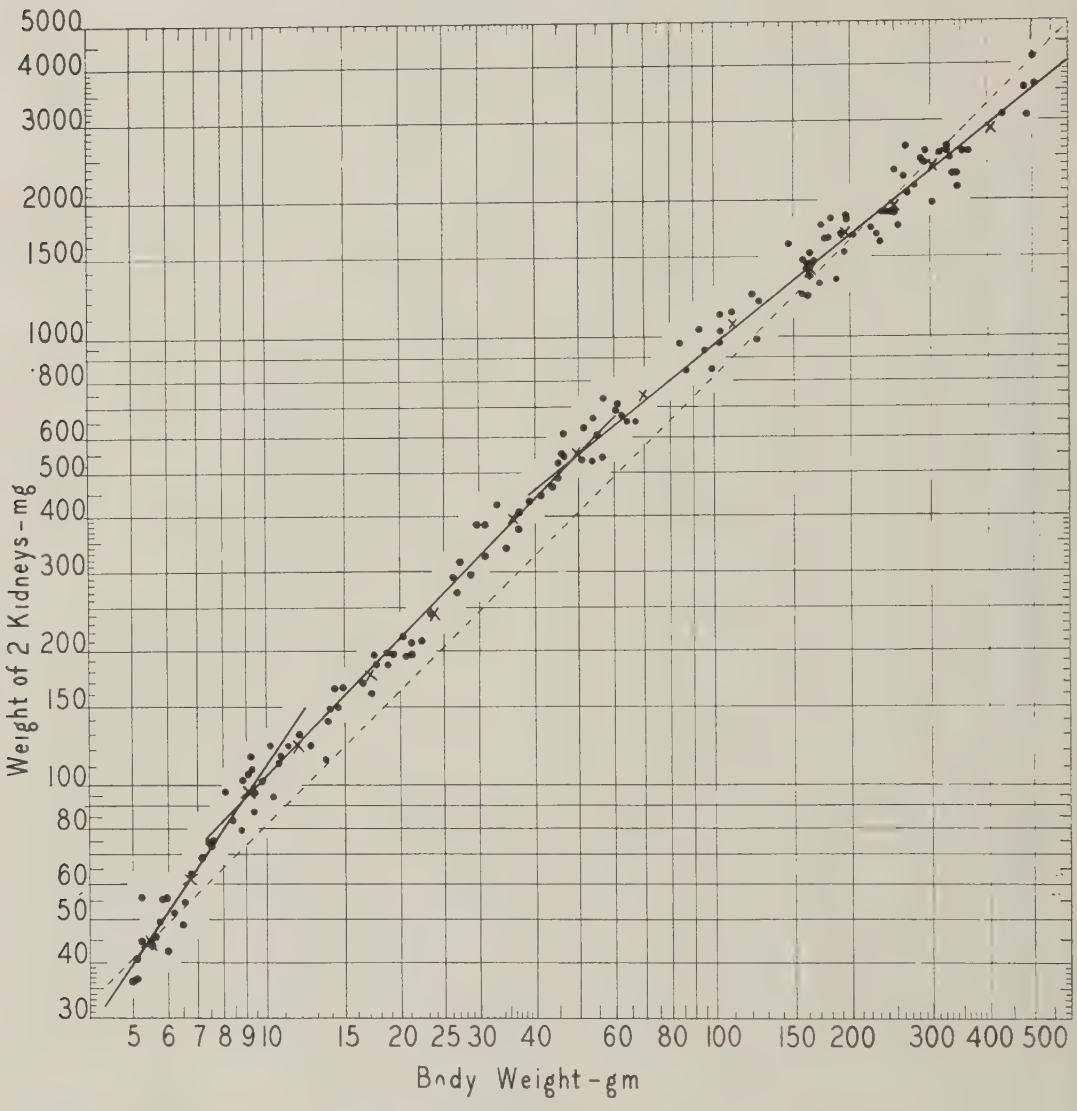

Fig. 1.

The weight of the kidneys plotted against body weight. Both axes are logarithmic. The points represent individual male rats; the crosses are the average values for 10 successive animals. The slope values indicate that during the first phase the percentage growth rate of the kidneys is 1.44 times that of the body; for the second and third phases the figures are respectively 1.02 and 0.81.

The dotted line (slope 1) indicates the course of relative growth if kidney and body weight remained proportional.

formation can be read from the graph if one considers that constancy of percentage value is represented graphically by a line of 45° to both horizontal and vertical axes such as the dotted line which represents 0.8%. The line of phase 2 approximates 1%.

When taken over the whole of postnatal growth organ weights usually show one or more breaks. On the other hand general body measures of the rat such as length or surface follow one continuous line throughout. The data of Carman and Mitchell[8] for instance show excellent adherence to a line with a slope of ⅓ for body length and

[8] Carman, G. G., and Mitchell, H. H., *Am. J. Physiol.*, 1926, **76**, 380.

a slope of ⅔ for body surface.

Summary. The growth in weight of the rat's kidneys in relation to body weight can be divided into 3 phases. Kidney weight expressed as % of body weight changes continuously but at 3 different rates.

15304

Kidney Hypertrophy in B Complex Deficiency.

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During a recent series of observations on gastric lesions in B-complex deficient rats our attention was attracted to the kidneys by the occurrence of an advanced case of hydronephrosis. Kidneys in the remaining rats of the group were inspected and weighed. The hydronephrosis may have been purely a chance phenomenon, since no more cases were noted. However all the other kidneys, though not abnormal in appearance, were considerably overweight. This seemed to be a point of some interest, especially since inanition controls showed no such enlargement.

The observations are presented individually in Fig. 1 in the form of a log-log plot of the weight of the 2 kidneys against the greatest body weight attained during the experimental period. The straight line represents the course of this relation for stock animals.[1] The B-deficient rats were placed at weaning (28 days, mean weight 64 g) on an ad libitum intake of the deficient diet and were weighed twice a week for a 3- to 4-week period. (Not all the rats survived the 4 weeks). The maximal weight attained was 68 g, final weight 50 g. Three B-deficient diets were used: 1049, composed of 12% purified casein (Labco), 4% salts, 0.1% carotene in oil (Smaco), 0.3% cod liver oil, 1.6% refined cottonseed oil, and corn starch to 100%; 1051, the same as 1049 but with crude casein substituted for the purified casein; and 1062, composed of 27% Labco casein, 3.4% salts, 2% celluflour, 0.2% carotene in oil, 0.25% wheat germ oil, 1.55% refined cottonseed oil, and cerelose to 100%. In all cases the diets contained 1.2% modified Wesson salt mixture (Ca and P free), 1.5% $CaCO_3$ and enough KH_2PO_4 to bring the total P of the diet to 0.41%. There appears to be no difference between the results on the 3 diets, and it is clear that the kidneys weighed about 1½ times what they would in a normal rat of the same body weight as that maximally attained by the B-deficient rats. The difference is highly significant statistically. If comparison were made with the final body weight the difference would of course be even greater.

The inanition controls were of 3 kinds. Control group 1 (partial inanition) was maintained at constant body weight for a 4-week period by limiting the intake of a normal diet. The actual mean weights were: initial 64, maximal 67, final 65. The diet was 1062, supplemented with B factors (1 mg each of thiamine and pyridoxin, 2 mg riboflavin, 4 mg each of calcium pantothenate and nicotinic acid, 200 mg choline per 100 g). Their kidneys appeared to be normal for body weight.

Control group 2 illustrates the effect of a continuous rapid weight loss. Stock rats (40 days, 118 g), in complete inanition, died in about 3 days after losing approximately 40% of their body weight. As the graph shows, their kidneys (represented by crosses) are 20% below the line. Since in these rats the maximal weight is the same as the initial (normal) weight, positions on the normal line in the region of A represent the kidney weights at the beginning of the experiment; compared with initial weight there has been an actual loss of 20% of kidney substance. The final body weights of these rats averaged 73 g; normal rats of this body weight have

[1] Stoerk, H. C., and Zucker, T. F., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**.

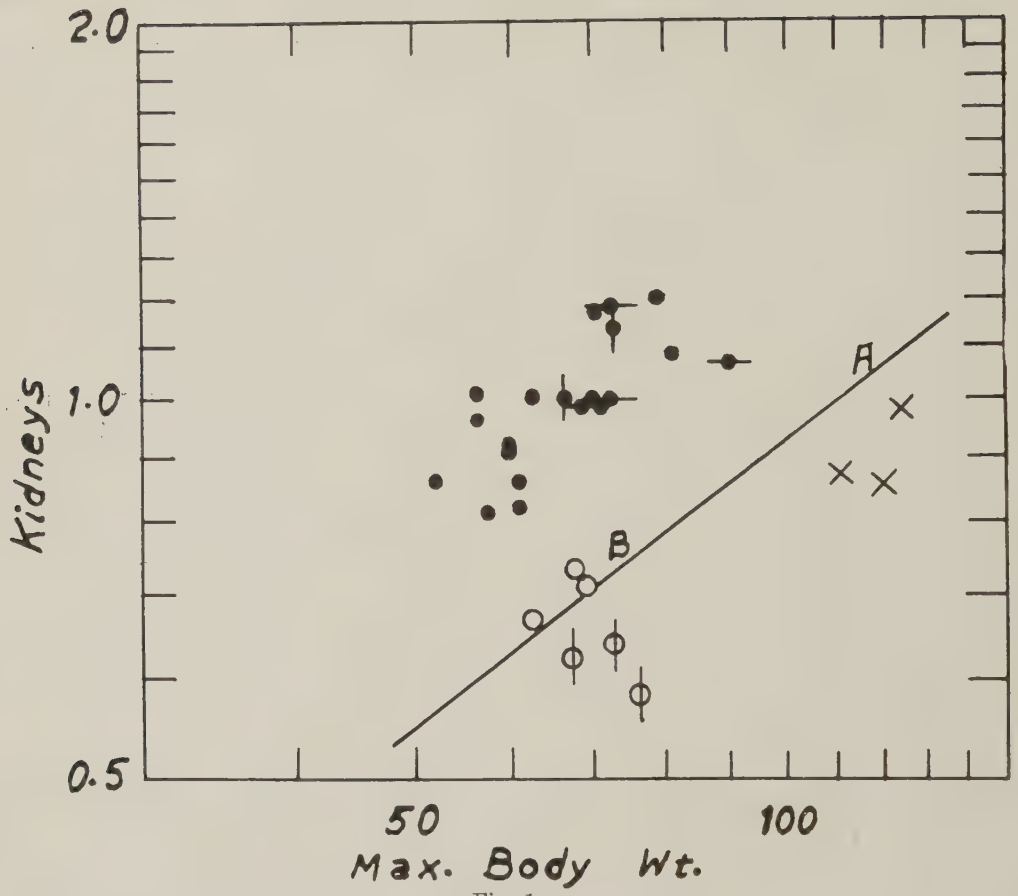

Fig. 1.

The scales are logarithmic. The line is the position of stock rats. A and B are regions of the graph discussed in the text.

The filled circles represent B-complex deficient rats (experimental); the plain ones had 12% vitamin-free casein, those with horizontal bars had 12% crude casein, and those with vertical bars had 27% vitamin-free casein.

The open circles represent rats on a restricted intake of a normal diet; the plain ones were given enough to maintain weight for 4 weeks (control group 1), and those with vertical bars (control group 3) were the food intake controls on the B deficient rats represented by filled circles with vertical bars.

The crosses represent 6-week-old rats which received no food (control group 2).

kidney weights on the line in the region of B, that is, distinctly smaller than the observed kidney weights. Evidently during rapid continuous weight loss the kidneys lose weight but less rapidly than the body as a whole. These differences, each about 20%, are statistically significant; the standard error of estimate for the normal data in the body weight region 50-150 g, is +5.8%, —5.5%.

The third group were pair-fed controls for the B-deficient rats; each received the same daily intake of the normal diet as its pair-mate on 1062 consumed spontaneously. The mean weights of this group were: initial 65, maximal 71, final 42. As with the starved rats, the kidneys are about as much too low for the maximal body weight as they are too high for the final body weight.

Summary. B-complex deficiency in 20 young rats resulted in kidneys which were 50% overweight. Nine rats which served as inanition controls showed no enlargement. Among the 3 types of food controls those which lost weight had kidney weights below the norm; where body weight was kept constant the kidney weights coincided with the norm based on stock diet animals.

15305

Observations on Rats Fed with Yellow A.B.

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In a previous communication[1] it was shown that the daily ingestion of large amounts of yellow O. B. (l-*O*-tolylazo-2-naphthylamine) fail to produce liver cirrhosis or tumors in rats over a period of 259 days of feeding and observation. This study was extended with Yellow A. B. (l-phenylazo-2-naphthylamine) because this oil-soluble coal tar dye is also extensively used in this country to color foodstuffs.* Yellow A. B. is somewhat related in structure to Butter Yellow (*p*-dimethylaminoazobenzene) which is one of the azo dyes which readily produces liver cancer in rats.[2] Because of this chemical relation, experiments were undertaken to determine whether or not the ingestion of large amounts of this coal tar dye, over a prolonged period, might give rise to liver cancer.

Experimental. Yellow A. B. (National Aniline and Chemical Company) was dissolved in cotton seed oil in the proportion of 6%. Twenty cc of this solution was mixed with 1000 g of coarsely ground, unpolished rice; 1000 g of unpolished rice containing 6% of dried whole milk; and thirdly 1000 g of screened Purina Dog Meal (a complete diet). All these 3 diets were supplemented with a small amount of fresh carrots daily. Unlimited water was allowed. Feeding on the various diets containing Yellow A. B. was continued for about 500 days; all rats still living were sacrificed and examined. Rats, male and female, (about 150 g body weight) used in this study were of Sherman stock.

The results showed that the daily ingestion of large amounts of Yellow A. B. in the above diets (each animal having consumed 8 to 12 mg of the dye daily) failed to produce liver tumors or cirrhosis in rats during 50 to 528 days' feeding.

Nutrition of young adult rats during ingestion of the Yellow A. B.-rice diet, Yellow A. B.-rice and Klim diet and the Yellow A. B.-normal diet, may be briefly summarized as follows:

With Yellow A. B.-rice diet, animals continuously lost weight until death occurred. Of 42 rats, 2 died during the first 50 days, 18 died between 51 and 100 days, 12 died between 101 and 150 days and remaining 10 died between 151 and 287 days. The addition of 6% of dried whole milk to Yellow A. B.-rice diet resulted in a distinct improvement in the health of rats but all of 20 rats died between 70 and 300 days. With the Yellow A. B.-normal diet, animals grew almost normally and were of general good appearance. Of the 22 rats on this last diet, 2 died between 50 and 100 days, 6 died between 101 and 150 days, 7 died between 300 and 502 days and 7 were sacrificed on 528 days.

The livers of animals fed the Yellow A. B.-rice diet with or without supplement of dried whole milk were pale and reddish or yellowish in color, while the livers of animals fed the Yellow A. B.-normal diet had normal color. There was no great change in the size or shape of the liver, which had smooth surfaces and histological examination revealed no evidence of tumors, bile duct changes, or abnormal regeneration of the ducts or liver cells. However, some sections (the livers of 8 out of 54 rats that lived more than 100 days) showed focal necrosis of the liver but this was not apparently related to any

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[1] Sugiura, K., PROC. SOC. EXP. BIOL. AND MED., 1942, **50**, 214.

* According to Mr. W. C. Bainbridge of H. Kohnstamm Company, New York, the annual consumption of these dyes in U. S. A. is about 44,000 lbs. Therefore, although the results of the experiments with these substances are negative, they are recorded because of general public interest in food dyes.

[2] Sugiura, K., and Rhoads, C. P., *Cancer Research*, 1941, **1**, 3.

one diet. The only visceral changes observed were those accompanying the terminal bronchopneumonia. No tumor was found in the visceral organs or elsewhere.

Conclusion. Under the condition of the above experiment, Yellow A. B. (l-phenyl-azo-2-naphthylamine) is not a carcinogenic substance.

The author wishes to express his appreciation to Dr. C. P. Rhoads for his interest and valuable advice.

15306 P

Effect of Choline on Blood and Tissues with Especial Reference to Cholesterol in Old Hens.*

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The lipotrophic activity of choline in experimental fatty cirrhosis of the liver, has been demonstrated by several groups of investigators.[1,2,3] The possibility of similar action on other types of cholesterol deposition in other tissue, particularly in atheromatous lesions in the aorta, has suggested itself. The naturally occurring human-like subintimal aortic atheromatosis as reported by Dauber[4a] was chemically studied in 3-year-old certified 260 to 320 egg R.P.O. sired hens and the average level of certain chemical components established.[4b] The effect of choline on this pathological chemistry presented itself as an important problem.

Steiner[5] first and then Bauman and Rusch[6] and about simultaneously Himsworth[7] reported that they had not been able to demonstrate any action of choline on the high blood, aortic or liver content of cholesterol in cholesterol-fed rabbits. Andrews and Broun[8] concluded that choline protected against atherosclerosis and Huber, Broun, and Casey[9] using lipocaic reported the prevention of atherosclerosis in cholesterol-fed rabbits. Steiner[10] intimated that choline may hasten reabsorption of atheromas.

The data from the original control series A of 26 3-year-old hens are set down for comparison.

A summer-autumn (S&A) and a winter-spring (W&S) choline fed series, each of 26 3-year-old hens individually caged and fed all that they would eat of a high fat and protein laying mash (Purina) and given, in addition, 0.5 g choline chloride daily. With the summer and autumn (S&A) choline series, a simultaneous control series B of 2 immediately sacrificed controls and 4 others that were sacrificed after 14 and 21 days; of the choline-fed series S&A, 6 were after 28; 4 after 35; 4 after 42; 2 after 56; 3 after 70; and 1 after 77 days of choline feeding. There were 6 complete simultaneous con-

* Supported by a grant from the Medical Research Department, Winthrop Chemical Co. Choline chloride was generously supplied by Merck & Co.

1 Best, C. H., and Rideout, J. R., *Am. J. Physiol.*, 1938, **67**, 122.

2 Dragstedt, L. R., *et al.*, *Arch. Int. Med.*, 1939, **64**, 1017.

3 McHenry, E. W., and Patterson, J. M. A., *Physiol. Rev.*, 1944, **24**, 128.

4 a. Dauber, D. V., *Arch. Path.*, 1944, **38**, 46; 4 b. Herrmann, George R., PROC. SOC. EXP. BIOL. AND MED., 1946, **60**.

5 Steiner, A., PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 231.

6 Bauman, C. A., and Rusch, H. P., PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 647.

7 Himsworth, H. P., *Acta. Med. Sc. and Supp.*, 1938, **90**, 158.

8 Andrews, K. R., and Broun, G. O., *J. Clin. Invest.*, 1940, **19**, 786.

9 Huber, M. J., Broun, G. O., and Casey, A. E., PROC. SOC. EXP. BIOL. AND MED., 1937, **37**, 441.

10 Steiner, A., PROC. SOC. EXP. BIOL. AND MED., 1939, **39**, 411.

TABLE I.
Controls and Summarized Data of Summer and Autumn Series Effect of Choline on Blood Constituents and Cholesterol in Tissues.

	NPN mg/100 ml	Urea N mg/100 ml	Glucose mg/100 ml	Serum protein mg/100 ml
Controls 26 (A) s. d.*	36.2 ± 6.4	19.0 ± 3.0	172 ± 48.4	4.9 ± 0.55
Controls simultaneous 6 (B)	34	17.9	222	5.3
Choline (S & A)† 20 (C) s. d.	37.4 ± 6.0	18.1 ± 8.5	206 ± 8.6	5.7 ± 1.3

Blood	Cholesterol	Total esters	Tissue cholesterol		
Control 26 (A) s. d.	mg/100 ml 248 ± 37	mg/100 ml	Aorta mg/250 mg 230 ± 43	Heart mg/250 mg	Liver mg/250 mg
Control 6 (B) s. d.	232.3 ± 20.9	153 ± 5.6	249 ± 29.4	271.3 ± 49.7	337 ± 54.1
Choline (S & A) 20 (C) s. d.	174 ± 25.4	121 ± 10.8	190 ± 29.2	205 ± 29.3	236 ± 92.9

* s. d.—Standard deviation.
† S & A—Summer and autumn series.

TABLE II.
Controls and Summarized Data of Winter and Spring Series Effect of Choline on Cholesterol Esters and Phospholipids in Blood and in Tissues.

	Cholesterol mg/100 ml		Phospho-lipids mg/100 ml	Cholesterol, total/esters mg/250 mg		
	Total	Esters		Aorta	Heart	Liver
Controls original old hens (A) 26 s. d.*	248 ± 37			230 ± 43.2		
Controls simulta-neous (B) 6 s. d.	232 ± 21	153 ± 5.6		249 ± 29	271 ± 49	337 ± 54
Control simulta-neous (C) 26 s. d.	271 ± 37	179 ± 19.2	9.6 ± 2.3	(4) e 230/176 ±45/±20	(4) e 288/233 ±80/±19	(4) e 342/240 ±62/±19
Choline (W & S)† (D) 26 (40-64 days)	179 ± 24	144 ± 9.2	10.1 ± 1.8	165/83 ±50/±24	198/147 ±57/±19	249/210 ±67/±25.6

* s. d.—Standard deviation.
† (W & S)—Winter and spring series.

trols. The blood and tissue chemistry of 20 showed definitely lowered total cholesterol and cholesterol ester values in blood and aorta compared with normal levels (Table I).

The winter-spring (W&S) choline-feeding series of 26 old hens were bled before choline

was started as a simultaneous control series C and 4 were sacrificed after 11 to 13 days for simultaneous tissue cholesterol control. Six hens were re-bled and sacrificed after 40 and 43 days; 10 after 47 and 49; 3 after 50 and 55 days; and 3 after 64 days of choline feeding. The data from the choline-fed series (W&S), sacrificed after 40 to 64 days, showed definite lowering of total cholesterol and cholesterol esters in the blood and in the aortae, as well as in the heart muscle and livers, as set down in Table II.

These data strongly suggest that the administration of 0.5 g choline daily to old hens on a purina laying mash diet for 4 to 10 weeks has little or no effect on the N.P.N., urea N., glucose, or serum protein levels.

It is, however, evident that the blood cholesterol total and ester levels are reduced and the organic phosphorous values slightly increased. The levels of these substances in the aorta, heart muscle, and liver are likewise changed. Cholesterol seemingly is mobilized from the blood and tissues of hens and metabolized under the influence of choline administration.

The generous cooperation of Mr. C. W. Carter of the Texas Agricultural Experimental Station is gratefully acknowledged.

15307

Japanese B Encephalitis Virus in the Blood of Experimentally Inoculated Chickens.*

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During the past 3 years studies on the Japanese B virus have been undertaken in this laboratory with 2 possibilities in mind; first, that United States combat and occupation troops would be entering epidemic areas and second, that the Japanese B encephalitis virus might be introduced on the North American mainland, particularly in the West. In such an event it would be of inestimable value to have as complete a knowledge as possible of the potential animal reservoirs and vectors.[1,2] The course of our investigations was influenced largely by our findings in epidemiological studies of the St. Louis and Western equine types of infection.[1,3]

In a separate paper we have reported studies in which 10 common species of Western North American mosquitoes were tested for their ability to act as vectors of Japanese B encephalitis virus.[4] Of the 10 species tested 7 were demonstrated to be laboratory vectors, and this included those species already proven to be important vectors of the Western equine and St. Louis encephalitis viruses.[1] The present paper reports tests of the ability of the chicken, a vertebrate host of the local endemic arthropod-borne encephalitides,[1,3] to act as a host for the Japanese B encephalitis virus.

Considerable difficulty has been encountered

* This investigation was carried out in collaboration with the Commission on Neurotropic Virus Diseases, Board for the Investigation and Control of Influenza and other Epidemic Diseases in the Army, Preventive Medicine Division, Office of the Surgeon General, U. S. Army; and under a contract, recommended by the Committee on Medical Research between the Office of Scientific Research and Development, and the University of California. Aided by a grant from the National Foundation for Infantile Paralysis.

1 Hammon, W. McD., and Reeves, W. C., *A. J. P. H.*, 1945, **35**, 994.

2 Hammon, W. McD., *Calif. and West. Med.*, 1944, **61**, 145.

3 Hammon, W. McD., Reeves, W. C., and Gray, M., *A. J. P. H.*, 1943, **33**, 201.

4 Reeves, W. C., and Hammon, W. McD., *J. Exp. Med.*, 1946, **83**, 185.

in obtaining the Japanese and Russian journals in which information on the vertebrate hosts of the Japanese B encephalitis virus has been published. According to those Japanese reports or abstracts available virus may be detected in the blood, saliva, urine and feces of human beings[5-7] and the blood of dogs, rats, horses, goats and sparrows,[5,6,8] even when no manifest disease is present. Russian workers have reported the isolation of virus from the blood of human patients[9] and birds[10] of endemic areas; and have reported a high incidence of immunity (35%) in normal horses of the affected areas. Shubladze[9] concluded that the natural reservoir of the virus is birds and horses. Smorodintsev[10] claimed that wild animals such as deer, wolves and birds constituted the most important reservoir.

It is difficult to interpret Russian and Japanese reports on isolation of this virus as they have announced that a virus encephalitis infection of mice is encountered in certain of their mouse colonies.[11,12] This virus cannot be readily differentiated from Japanese B either clinically, serologically or by animal susceptibility range. Some Japanese and Russian claims of virus isolations are based on the demonstration of virus after 2 to 6 "blind" serial passages of mouse brain. When such has occurred, the work cannot be accepted as conclusive. Unfortunately experimental details are not available in many instances, in which virus isolation is reported, and we are therefore unable to draw satisfactory conclusions.

Methods and Materials. The technics employed in these studies are in general the same as those reported in our previous studies on St. Louis[13] and Western equine viruses[14] in the blood of experimentally inoculated chickens. The virus employed was the Nakayama strain, obtained from Lt. Col. A. B. Sabin, M.C. A.U.S. This virus had been thoroughly mouse brain-adapted by repeated passage. This adaptation was probably a disadvantage but no freshly isolated virus strains were available. Our work with domestic viruses had all been with strains recently isolated from mosquitoes.

All the chickens used were Hampshire reds, incubator hatched, and guarded from mosquitoes and other blood-sucking arthropods till used at from 2 to 3 months of age.

Mice used for the detection of virus were either the Webster Swiss, or the Rockefeller Institute albino strain. They were employed at 3 to 4 weeks of age.

Experiment 1. The first experiment was in the nature of a preliminary trial to determine if chickens would develop a viremia following subcutaneous inoculation of a relatively large dose of virus. Four chickens were inoculated subcutaneously with 1.0 cc of a 10^{-2} dilution of mouse brain virus. The results of the chicken serum and organ tests in Exp. 1 are presented in Table I. It will be noted that virus appeared in the serum 24, 48, and 72 or 96 hours following inoculation of the chickens.

The brain from the single mouse which developed encephalitic signs following the inoculation of a 24-hour serum sample from chicken 934 was passed to 3 mice all of which developed a typical encephalitis. In all other instances when virus was detected, the quantity was adequate to kill all 5 mice

5 Mitamura, T., Kitaoka, M., Watanabe, S., Hosoi, T., Tenjin, S., Seki, O., Nagahata, K., Jo, K., and Shimigu, M., *Tr. Soc. path. jap.*, 1939, **29**, 92.

6 Mitamura, T., Kitaoka, M., Watanabe, Z., and Tenjin, S., Record 13th Meeting United Assn. of Microbiology of Japan, 1939.

7 Mitamura, T., Kitaoka, M., and Watanabe, Z., *Tokyo J. Med.*, 1939, No. 3143, 1880.

8 Kawamura, R., Kodama, M., Ito, T., Yasake, T., and Kobayakawa, Y., *Kitasato Arch. Exp. Med.*, 1936, **13**, 281.

9 Shubladze, A. L., *J. mikrobiol. epidemiol. immunobiol.*, 1943, **1-2**, 87, as reviewed by Rosenthal, L.; *Am. Rev. Soviet Med.*, 1944, **2**, 166.

10 Smorodintsev, A. A., *J. Mikrob. epid., immun., Moskva*, 1942, **11-12**, 67.

11 Kawamura, R., Kasahara, S., Miyata, T., Veda, M., and Yamada, R., *Kitasato Arch. Exp. Med.*, 1940, **17**, 38.

12 Smorodintsev, A. A., personal conversation with W. McD. H.

13 Hammon, W. McD., Reeves, W. C., and Izumi, E. M., *J. Exp. Med.*, 1946, **83**, 175.

14 Hammon, W. McD., and Reeves, W. C., *J. Exp. Med.*, 1946, **83**, 163.

TABLE I.

Exp. 1. December 1944. Results of Tests for Virus Made on the Serum of Chickens Inoculated Subcutaneously with 1.0 cc of Japanese B Encephalitis virus, Nakayama Strain, in a 10^{-2} Dilution.

	Period after inoculation when bled or autopsied and serum or organs tested for virus			
Chicken	Serum 24 hr	Serum 48 hr	Serum 72 or 96 hr†	Spleen and brain* 40 days
933	5/5‡		0/5	0/5
934	1/5		5/5	0/5
931		5/5	0/5	0/5
932		0/5	0/5	0/5

* Spleens and brains from all 4 chickens pooled and one set of 5 mice inoculated.

† Due to loss of labels the 72-hour and 96-hour bleedings were confused.

‡ Numerator indicates number of mice that died and the denominator the number inoculated.

inoculated.

After 40 days, all 4 chickens were sacrificed, the spleens and brains were pooled and tested for virus with negative results.

Experiment 2. Having demonstrated in Exp. 1 that chickens would develop a viremia following subcutaneous inoculation of large amounts of virus, Exp. 2 was performed to determine in greater detail the length of time during which virus would persist in the serum and the spleen of chickens inoculated subcutaneously with a small amount of virus. A small amount was used because it was wished to duplicate what might occur naturally from a mosquito bite. Eight chickens were inoculated with 0.3 cc of a 100 LD-50 dilution of virus, as determined by titration intracerebrally in mice (1:9,000,000 dilution of mouse brain). Bleedings were begun 24 hours after inoculation and continued every 12 hours up to 120 hours and every 24 hours from 120 to 192 hours after inoculation. Two chickens were bled at each period. Each serum was tested by intracerebral inoculation of 5 mice (Table II). When only one of the 5 mice succumbed a frozen portion of the same serum was inoculated into 5 other mice for confirmation. If 2 or more of the 5 mice succumbed a frozen portion of the serum was titrated in 10 fold dilutions. The death of a single mouse in a group of 5 inoculated with undiluted serum, we do not accept as proof of the presence of virus. Virus was apparently isolated (at least 2 of 5 mice inoculated died at the proper interval), on at least one occasion from the undiluted serum of 5 of the 8 inoculated birds. Virus was demonstrated from at least one of the 2 chickens tested at 48, 72, 84, 96, 144 and 168 hours after inoculation. At 144 and 168 hours isolations were made from the serum of both birds tested. As an indication that virus titers were low, it may be observed that in no instance was virus demonstrated (by the death of more than one mouse) in a 1:10 dilution of the serum, nor were all mice killed by the undiluted serum as had occurred regularly in Exp. 1. Virus was not isolated from the spleens of any of the chickens in tests made at 120, 144, 168 and 192 hours.

Miscellaneous experiments to demonstrate infectivity of chicken blood. A single attempt was made to infect a chicken by the bite of infected mosquitoes (*Culex pipiens* Linn.). This experiment was successfully concluded; virus was demonstrated in the chicken's serum 48 and 96 hours after the bite of 4 of the infected mosquitoes. Five out of 5, and 4 out of 5 inoculated mice succumbed respectively from these sera.

Two other chickens, employed in a mite feeding experiment, were bled following subcutaneous inoculation of 0.2 cc and 1.0 cc respectively, of a 10^{-3} dilution of mouse-brain-virus. The serum of the chicken inoculated with the smaller dose, when taken 48 hours after injection, killed all 5 inoculated mice and the serum of the latter, tested at 48 and 72 hours failed to kill any of the mice.

Two attempts to infect mosquitoes from inoculated chickens were unsuccessful. In one

TABLE II.

Exp. 2, 4-5-45. Results of Tests for Virus Made on the Sera and Spleens of Chickens Inoculated Subcutaneously with 0.3 cc (100 Mouse LD-50 dilution) of Japanese B Virus.

Chickens	Virus isolation at some time	Hours after inoculation when bled or autopsied and serum or spleen dilution tested for virus: 24 hr Serum fresh undil.	36 hr S* F† U‡	48 hr S F U	48 hr S Fro§ U	48 hr S Fro§ 10-1	60 hr S F U	72 hr S F U	72 hr S Fro U	72 hr S Fro 10-1	84 hr S F U	84 hr S Fro U	84 hr S Fro 10-1	96 hr S F U	96 hr S Fro U	96 hr S Fro 10-1	96 hr S Fro 10-2
830	0	0/5						0/5									
831	+	0/5						2/4	1/5	0/5							
832	+		0/5								0/5						
833	+		0/5								2/4	0/5	0/5				
834	+			2/5	1/4	0/5								4/5		1/5	0/5
835	+			1/4	0/5									1/4	0/5		
836	0						0/5										
837	?						0/4										

Chickens	Virus isolation at some time	Hours after inoculation when bled or autopsied and serum or spleen dilution tested for virus: 108 hr S F U	108 hr S Fro U	120 hr S F U	120 hr Spleen F 10-1	144 hr S F U	144 hr S Fro 10-1	144 hr S Fro 10-2	144 hr Spleen F 10-1	168 hr S F U	168 hr S Fro U	168 hr S Fro 10-1	168 hr S Fro 10-2	168 hr Spleen F U	192 hr S F U	192 hr Spleen F 10-1
830	0			0/5	0/5											
831	+				1/5											
832	+					4/5	0/5	0/5	0/5							
833	+					4/5	1/5	0/5	0/5							
834	+									3/5	0/5	0/5		0/5		
835	+									2/5	2/5	1/5	0/5	0/5		
836	0	0/5													0/4	0/5
837	?	1/5	0/5												0/5	0/5

* S = Serum.
† F = Fresh.
‡ U = Undiluted.
§ Fro = Frozen.

instance 42 mosquitoes fed and in the other 58. Both chickens had been inoculated 48 hours previously with 1.0 cc of a 10^{-3} dilution of mouse brain virus. Five out of 5 and 2 out of 5 mice, respectively, died from inoculation of serum from these chickens at the time of mosquito feeding.

Conclusions. As a result of inoculating Japanese B encephalitis virus subcutaneously in chickens, virus can frequently be detected in the serum 24 hours to 7 days later. Even the subcutaneous inoculation of minute amounts of virus frequently resulted in viremia. In addition, it was demonstrated that following the bite of 4 infected mosquitoes infection with viremia occurred in the one chicken used. In these experiments, when employing a "brain-adapted" strain of virus and 2- to 3-months-old chickens, the titer of virus in the serum was relatively low. It was lower using this agent, than in similar experiments with strains of St. Louis and Western equine viruses recently isolated from mosquitoes. In 2 test feedings on chickens inoculated with Japanese B virus no mosquitoes became infected, but we feel that no conclusions should be drawn from such a limited test. Virus did not persist in the spleen of inoculated chickens over 8 days nor in the brain over 40 days (not tested earlier). These experiments do not conclusively demonstrate that the chicken can or cannot be a source of Japanese B virus infection for mosquitoes, but indicate that such a possibility exists. We feel that chickens and other birds should be considered potential sources of mosquito infection and that they deserve further study with recently isolated strains of virus.

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Studies on Scrub Typhus. II. Preparation of Formalinized Vaccines from Tissues of Infected Mice and Rats.*

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Following a number of unsuccessful attempts to prepare a potent inactive vaccine against scrub typhus (see references cited in [1]) materials which showed promise were obtained in 3 laboratories[1,2,3] almost simultaneously. The first of these vaccines[1] was prepared by an adaptation of the rodent lung method used by Castaneda[4] and by Durand and Sparrow[5] for the cultivation of rickettsiae of murine and epidemic typhus; a tissue culture method was used for the second type of vaccine;[2] and rodent tissues for the third.[3] The present report describes hitherto unpublished data on the preparation of a formalinized vaccine from the lungs and spleens of rodents infected by the intravenous route.

* Most of the experimental work discussed here was done in the Virus Division, First Medical General Laboratory, U. S. Army. It was reported to the Director, U. S. A. Typhus Commission, and certain workers in the field in confidential communications listed in reference.3

1 a. Fulton, F., National Institute for Medical Research, London, 21 June 1944; b. Fulton, F., and Joyner, L., *Lancet*, 1945, **2**, 729.

2 a. Plotz, H., Bennett, B. L., Reagan, R. L., Bell, E. J., Hamilton, H. L., and Snyder, M. J., Report to the Director, USA Typhus Commission, 23 September 1944; b. Plotz, H., Bennett, B. L., and Reagan, R. L., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 313.

3 Smadel, J. E., Rights, F. L., and Jackson, E. B., confidential reports to the Director, USA Typhus Commission, submitted 2 October 1944 and 19 June 1945, respectively.

4 Castaneda, M. R., *Medicine*, Mexico, 1938, **18**, 607; *Am. J. Path.*, 1939, **15**, 467.

5 Durand, P., and Sparrow, H., *Arch. Inst. Pasteur Tunis*, 1940, **29**, 1.

Materials and Methods. The majority of the materials and methods employed in the present work were similar to those which have been described in detail in a recent paper.[6] These included methods of maintaining the tsutsugamushi organisms, of determining infective titer, and of performing complement fixation tests. In addition to the Imphal No. 8 and Calcutta strains of *R. orientalis* used previously, the Karp[2b,7] and Kostival[8] strains were employed in certain of the current tests. Scrub typhus vaccines were prepared in a manner identical with that described[6] at length for making "crude antigens." In brief, the method was this: white mice, white rats (weight 125-150 g), or cotton rats were injected intravenously with 0.5, 2.0, or 1.0 cc, respectively, of a 10% suspension of infected yolk sac which contained about 10^8 MLD of *R. orientalis* on the basis of intraperitoneal titration in mice. Lungs and spleens were harvested during the fourth or fifth day when the animals were moribund or recently dead. After the tissues were ground in a mortar with alundum, 10% suspensions were prepared in physiological saline solution and these were freed of large particles by centrifugation in the horizontal machine at 2,000 r.p.m. for 5 minutes. A portion of the supernatant fluid was immediately titered for infectivity. The remainder was promptly treated with sufficient USP formaldehyde solution and merthiolate to bring the final concentrations to 0.1 and 0.01%, respectively. The formalinized suspensions were stored at + 5°C for one to 6 weeks and then employed in vaccination experiments. The amounts of complement fixing antigen of scrub typhus in most of the materials used as vaccines were determined by titration. These data are given in Table V of the first paper in this series.[6]

Groups of 24 to 70 mice were vaccinated with the materials under investigation. Each animal received 3 intraperitoneal injections of 0.5 cc amounts of vaccine given at 5-day intervals. All mice dying during the 24 days following the first injection were autopsied; none showed evidence of scrub typhus infection. Two weeks after the last injection, treated mice were tested for resistance to infection with the homologous strain of scrub typhus. Such challenge tests were performed in the following manner. Serial 10-fold dilutions of fresh infectious yolk sac were prepared in a mixture containing physiological saline solution and 10% normal horse serum and were kept in an ice bath until the challenge inoculation was completed. Six to 10 vaccinated mice were injected intraperitoneally with 0.2 cc amounts of one of the dilutions of infectious suspension. Depending on the available number of vaccinated mice, the dilutions of yolk sac for challenge were so chosen that they should contain a sufficient number of MLD's of *R. orientalis* to cover all or part of the range from 1 to 10^7. A group of normal control mice was injected with each dilution of the challenge material immediately after the test animals had received portions of the same suspension. Control mice used in these experiments were of the same strain and weight as the vaccinated mice. Infected mice were observed for 21 days and deaths recorded.

The resistance of treated mice to infection with *R. orientalis* was estimated in the following manner. The 50% end-point method of calculating infective titer was applied to the data obtained when groups of treated and control mice were challenged with serial 10-fold dilutions of infectious material. In those experiments in which some mice survived among the group that received the most concentrated challenge material employed it was assumed, for the purpose of calculating the end-point, that if a still more concentrated inoculum had been used then all of the mice would have succumbed. Thus, in Table I, Exp. 3, Vaccine 35 Spleen, only one of 8 mice died when challenged with a 10^{-4} dilution of material; nevertheless, it was assumed that all 8 mice would have died if they had received a 10^{-3} dilution of the same challenge

[6] Smadel, J. E., Rights, F. L., and Jackson, E. B., *J. Exp. Med.*, 1946, **83**, 133.

[7] Bengtson, I. A., *Pub. Health Rep.*, 1945, **60**, 1483.

[8] Blake, F. G., Maxcy, K. F., Sadusk, J. F., Jr., Kohls, G. M., and Bell, E. J., *Am. J. Hyg.*, 1945, **41**, 243.

TABLE I.
Resistance of Vaccinated Mice to Scrub Typhus.

Exp. No.	Immunized with vaccine number	Dilution of challenge inoculum 10-2	10-3	10-4	10-5	10-6	10-7	10-8	10-9	Titer 50% lethal (log)	Immunity index (log)
1 (Imphal)	Untreated					4/5	4/5	5/5	1/5	—8.4	
	Mouse 26 Lung				2/5	3/6	1/6	3/6		—6.1	2.3
	Mouse 26 Spleen				1/5	1/6	3/6	1/6		—5.4	3.0
2 (Imphal)	Untreated						4/4	3/3	0/4	—8.5	
	Mouse 27 Lung				4/6	1/5	0/5			—5.4	3.1
3 (Imphal)	Untreated					7/8	7/8	4/8	1/8	—7.9	
	Cotton rat 32 Lung				3/7	2/6	1/6	0/6		—5.3	2.4
	Cotton rat 34 Lung				2/6	1/6	1/5	1/5		—5.2	2.7
	White rat 35 Lung		4/8	2/8	5/9	1/9	3/8	0/8		—4.2	3.7
	White rat 35 Spleen			1/8	2/8	3/8	0/8	0/8		—3.9	4.0
4 (Karp)	Untreated	10/10	10/10	8/10	10/10	10/10	9/10	10/10	3/10	—8.5	
	White rat AMS 2 Lung & spleen	9/10	6/10	5/10	0/10	3/10	1/10	1/10		—4.0	4.5

Denominator indicates size of group challenged.
Numerator indicates number of mice in each group that died from the challenge inoculation.

inoculum. The immunity index value for a vaccine represented the difference between the infective titers determined in control and vaccinated mice and was expressed as a positive logarithm.

Results. The results summarized in Table I indicate that, under the conditions of these experiments, mice develop resistance to infection with *R. orientalis* following vaccination with formalinized suspensions of tissues of mice, white rats, or cotton rats that die from scrub typhus. It is apparent from the data presented that resistance varied among individual mice in a group which received a given vaccine.† For example, in Table I, Exp. 1, 3 of the 6 mice immunized with spleen vaccine succumbed when challenged with approximately 25 MLD's of *R. orientalis* while 4 of 5 survived 2500 MLD's. In order to overcome difficulties caused by such individual variation, the number of mice in each group was increased and the range of dilutions employed in the challenge inoculation was broadened. The type of procedure now employed in this laboratory for assay of vaccines prepared from lung and spleen of infected white rats, as well as from tissue cultures of *R. orientalis*,[2] is illustrated in Table 1, Exp. 4. It will be noted that the immunity index in this experiment was 4.5; in other words, the mice resisted about 32,000 MLD's of rickettsiae. It is also apparent from the tabular data that the maximal extent of the immune response elicited in mice was not determined for certain of the vaccines prepared from mice and cotton rats. This was because the lowest dilution of challenge material employed did not kill the majority of the vaccinated mice which received it. Consequently, even though the values of the immunity indices were less for vaccines prepared from these 2 species than for those derived from white rats, no conclusions are warranted regarding comparative immunogenic properties of vaccines prepared from the 3 species.

The immunological specificity of the re-

† Our observations, like those of others,[8] indicate that mice which survive inapparent infection with scrub typhus consistently resist intraperitoneal challenge with large doses of *R. orientalis*.

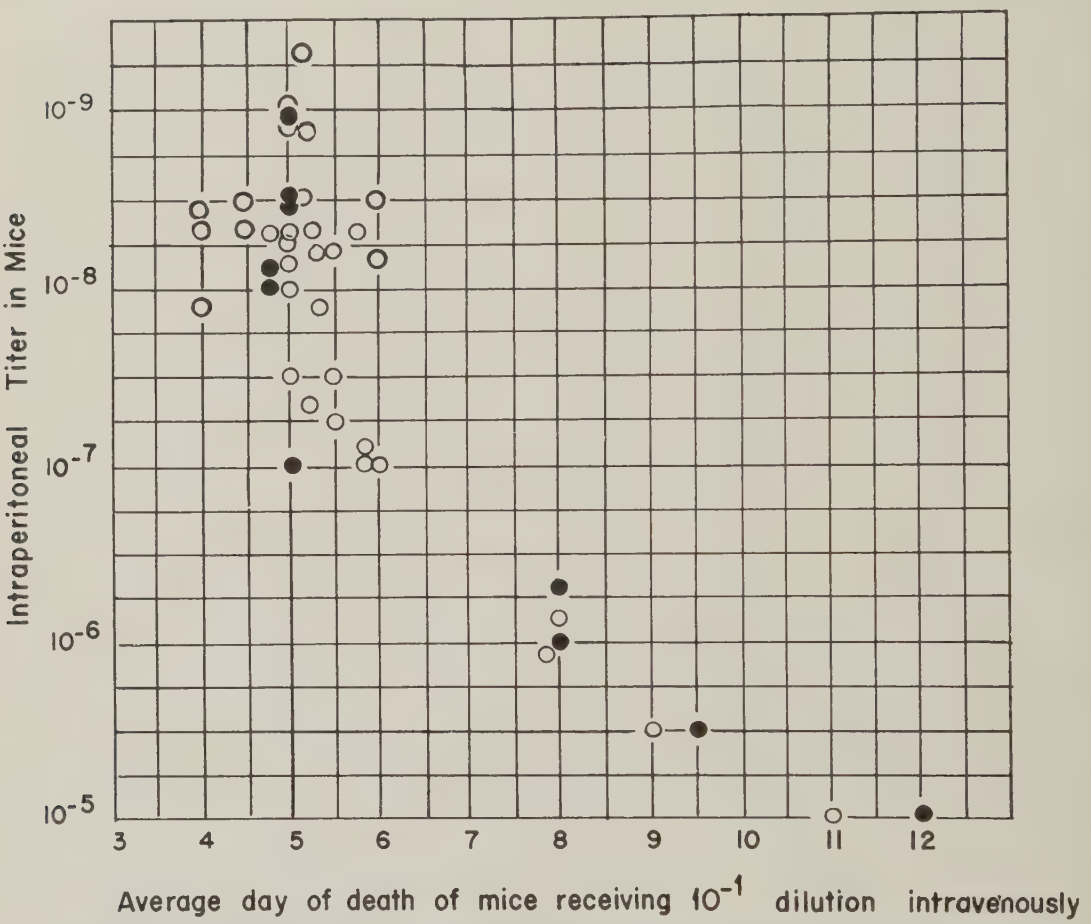
sistance induced in mice by the scrub typhus vaccines is evident from the following observations. Mice injected intraperitoneally on 3 occasions with 0.5 cc amounts of "normal tissue vaccines" developed no appreciable resistance to infection by the intraperitoneal route with *R. orientalis*. Normal tissue vaccines consisted of formalinized 10% suspensions of lungs of uninoculated mice or formalinized ether extracted 10% suspensions of yolk sacs of uninoculated 12-day-old embryonated eggs. In one experiment with normal mouse vaccine the titer of a given suspension of infectious material was $10^{-7.0}$ in unvaccinated mice and $10^{-7.3}$ in vaccinated animals; in a similar experiment with normal yolk sac vaccine the values were $10^{-8.2}$ in the control and $10^{-8.0}$ in the vaccinated group. Similar results with normal tissue vaccines have been reported by other workers.[1,2] Tests on 10 impotent scrub typhus vaccines gave further evidence that the degree of resistance obtained in the experiments summarized in Table I was significant. The immunity index of each of these vaccines was in the neighborhood of 1.0; in other words, such vaccinated mice resisted not more than 10 MLD's of rickettsiae. These 10 impotent vaccines were of 2 types: (1) ether extracted formalinized suspensions of infected yolk sacs, and (2) suspensions of mouse tissue with low infective titers (less than $10^{-7.0}$) before inactivation.

Potent scrub typhus vaccines have been prepared from rodent tissue which were highly infectious. The suspension of pooled lung and spleen used to prepare vaccine AMS 2 listed in Table I titered $10^{-8.0}$. The infective titers of the tissues employed as starting material for the other vaccines mentioned in Table I ranged from $10^{-7.5}$ to $10^{-9.0}$, see Table V of the first article in this series.[6] As already indicated, the immunity indices obtained for 8 lots of mouse vaccine that had titers of 10^{-5} to 10^{-7} before inactivation were never greater than 1.5 and in most instances the values were about 1.0. Therefore, vaccines with definite immunogenic properties were prepared only from tissues with infective titers in the neighborhood of 10^{-8}.

Attempts to estimate the immunizing capacity of a scrub typhus vaccine on the basis of its content of complement fixing antigen have not been encouraging to date. While potent vaccines contained sufficient amounts of such antigen to give titers of 1/3 to 1/6 (Table V, reference [6]), nevertheless, a number of the unsatisfactory vaccines also contained the antigen in some quantity. Furthermore, the serological titer did not correlate sufficiently closely with the infective titer to provide a means of estimating the latter over the range from $10^{-7.5}$ to $10^{-9.0}$.[6]

An assay for determining the immunizing capacity of a scrub typhus vaccine is a lengthy and expensive procedure. In the course of the present work, certain criteria have been adopted which provide a series of checks on a vaccine during its preparation. Since these criteria have been employed, final assay has been avoided on a number of vaccines which would probably have given immunity indices of less than 3.0 and, more important, good vaccines have been produced consistently. The following points are now observed. A strain of *R. orientalis* is not employed for the preparation of white rat vaccines until it is sufficiently well adapted to growth in eggs to provide, with fair regularity, yolk sacs which are infective for mice at a dilution of 10^{-8}. Fortunately, an estimation of the infective titer of each yolk sac inoculum can be obtained rather rapidly by determining the average day of death of mice injected intravenously with 0.5 cc amounts of a 10% suspension of the egg material (Fig. 1). Therefore, at the time white rats are injected intravenously with an infectious yolk sac suspension a group of 4 to 6 mice is injected by the same route with 0.5 cc amounts of the material. If the rats and mice die during the fourth or fifth day then the lung and spleen tissues of the rats are harvested, pooled and titered intraperitoneally in mice. The suspension of rat tissue is formalinized and stored at 5°C during the 21 days required for titration. If the infective titer is 10^{-8} or greater, the vaccine is considered worthy of a final assay for immunogenic activity. Since this system was instituted, 4 successive vaccines prepared

Fig. 1.
Correlation of Infective Titer of Scrub Typhus Yolk Sacs with Time of Death of Mice Injected Intravenously with a 10^{-1} Dilution.



Open circles in the above graph represent data obtained with 9th to 42nd yolk sac passages of Imphal S strain while solid circles indicate results with 13th to 30th yolk sac passages of the Calcutta strain, respectively.

from white rats infected with Karp or Kostival organisms have been tested and found to have immunity indices of 3.3, 3.3, 4.0, and 4.5. Two batches of vaccine prepared during this period were not assayed because they failed to meet one of the preliminary criteria.

Discussion. Although white rats were used in the preparation of the most potent scrub typhus vaccines made during the present studies, infected mice and cotton rats also supplied material for immunologically active vaccines. The white rat offers a number of advantages over the other 2 species of rodents for the preparation of scrub typhus vaccine on even a laboratory scale; one of the more important is the greater yield of infected tissue. For example, from the 6 white rats used for Vaccine 35, 12.7 g of lung and 7.9 g of spleen were harvested and these tissues had infective titers of $10^{-9.0}$ and $10^{-8.3}$, respectively. In contrast, the 6 cotton rats employed for Vaccine 34, which received the same inoculum used for the rats of Vaccine 35, yielded only 5.7 g of lung and 0.7 g of spleen which had infective titers of $10^{-8.0}$ and $10^{-7.8}$. Thus, with the same expenditure of effort, 20.6 g of starting material was obtained from white rats compared with 6.4 g from cotton rats. Vaccines prepared during the

past few months have consisted of pooled lung and spleen tissue of infected white rats; 30 to 35 cc of vaccine were obtained regularly from each animal.

Fulton and Joyner[1] prepared scrub typhus vaccine from lungs of cotton rats infected by the intranasal route with suspensions of mouse lung rich in *R. orientalis*. The vaccines produced by this method and by ours were comparable immunizing agents. Thus, the vaccine used in the experiment summarized in their Table VII has an immunity index of 3.7 according to our manner of expressing immunogenic activity. Certain difficulties encountered in the technic of these authors for preparing scrub typhus vaccine have been adequately emphasized.[1,9] The method we have used avoided or minimized several of these difficulties. In the first place, bacterial sterility of seed inoculum was no problem when yolk sac material was used. Secondly, the intravenous route of inoculation of dangerous material was relatively safe and simple compared with the intranasal route. Finally, white rats were easier to obtain and handle than cotton rats. These advantages are apparent now. However, the British workers were the first to report on the preparation of a scrub typhus vaccine with some immunological activity and we agree with their statement that "in wartime—expense and effort are secondary considerations, . . ."[1]

It is apparent from the accompanying paper by Plotz and his coworkers[2] that scrub typhus vaccine can be prepared from tissue cultures and that such vaccines are as potent as those made from white rats. It was, and still is, our opinion that any one of the 3 methods, *i.e.*, intranasal inoculation of cotton rats, growth in tissue cultures, or intravenous injection of white rats, could be used for the production of appreciable quantities of vaccine but that each would present serious technical difficulties for commercial application. It has been adequately shown that the use of yolk sacs infected with the rickettsiae of epidemic typhus has proved suitable for large scale manufacture of a potent vaccine against this disease. However, the application of these methods to the production of scrub typhus vaccine remains to be accomplished.

Summary. Formalinized vaccines prepared from lungs or spleens of white mice, cotton rats, and white rats infected with *R. orientalis* are capable of protecting mice against infection with scrub typhus. White rats are the animals of choice for the preparation of vaccines by the method described.

[9] Buckland, F. E., Dudgeon, A., Edward, D. G. F. F., Henderson-Begg, A., MacCallum, F. O., Niven, J. S. F., Rowlands, I. W., Van den Ende, M., Bargmann, H. E., Curtis, E. E., and Shepherd, M. A., *Lancet*, 1945, **2**, 734.

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Preparation of an Inactivated Tissue Culture Scrub Typhus Vaccine.

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The occurrence of scrub typhus in combat areas stimulated research on the development of a scrub typhus vaccine.[1] Three such vaccines have been described. The first of these was prepared from the lungs of intranasally infected cotton rats,[2] the second from agar

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[1] Blake, F. G., Maxcy, K. F., Sadusk, J. F., Jr., Kohls, G. M., and Bell, E. J., *Am. J. Hyg.*, 1945, **41**, 243.

[2] a. Fulton, F., National Institute for Medical Research, London, 21 June 1944; b. Fulton, F., and Joyner, L., *Lancet*, 1945, **2**, 729; c. Buckland, E. E., Dudgeon, A., Edward, D. G. F. F., Henderson-Begg, A., MacCallum, F. O., Niven, J. S. F.,

tissue cultures,[3] and the third from the lungs or spleens of intravenously infected white mice, cotton rats or white rats.[4] The present paper describes certain results which have already been reported in a restricted communication[3] as well as unpublished data, on the preparation of an inactivated vaccine from agar tissue cultures.

Materials and Methods. The peritoneal exudate of mice infected with the Kostival[1] or Karp† strains were used to initiate yolk sac cultures. The early yolk sac passages were rather poor in rickettsiae as judged by stained preparations (fix in methyl alcohol 3 minutes, stain with 2 cc of Giemsa in 58 cc of M/50 buffer solution pH 7.2 for 30 minutes, decolorize with acetone 3 seconds, water). However, by selection and subsequent passages the yolk sacs became richer so that after the 40th passage of the Kostival strain, and the 30th passage of the Karp strain, yolk sacs were occasionally found that appeared as rich in rickettsiae as yolk sacs infected with the Breinl epidemic typhus strain. After 55 passages of the Kostival strain about 40% of the yolk sacs were considered rich, this increased to about 80% after 82 passages but no additional improvement occurred up to the present (122 passages). Optimal growth of *R. orientalis* in the yolk sac was found to occur when 5- or 6-day fertile hens' eggs were inoculated and maintained at 35°C for 8 days. The embryos usually died on the 10th day under these conditions. The 50th yolk sac passage of the Kostival strain, when titered intraperitoneally, was infectious for mice at a dilution of 10^{-7} and the 60th passage at $10^{-8.3}$. The 36th passage of the Karp strain had an infective titer of $10^{-9.1}$. Yolk sacs that were rich in *R. orientalis* were selected to initiate agar tissue cultures. Seed materials from the 50th to 61st passage of the Kostival strain and of the 36th passage of the Karp strain were used in preparing the vaccines mentioned in Table I.

The agar tissue culture technic used by Zinsser, Plotz and Enders[5] for the preparation of an epidemic typhus vaccine was employed in the present studies. Ten- or 11-day-old normal chick embryos were removed sterilely and after excising the eyes, the embryos were passed through the Fisher press in order to obtain pieces of embryo tissue of approximately the same size. The inoculum was prepared as follows: infected yolk sac was ground in a mortar with alundum and a 20% suspension was made using the infected yolk fluid as diluent. About 7 cc of normal chick cell suspension and 3 cc of yolk sac material were thoroughly mixed, using a 10 cc pipette with a broken end. This mixture was placed in the ice box (+ 4°C) for 20 to 30 minutes and then 2 cc of it were delivered to each flask and evenly spread over the surface of the agar with a bent glass sterile rod. After carefully corking the flask with a rubber stopper, the flasks were incubated at 35°C for 8 days. At this time the Giemsa stained preparations in a relatively large number of flasks showed the cultures to be rich in intra- and extracellular rickettsiae. In contrast to the appearance of the rickettsiae in yolk sac preparations, the organisms in the agar tissue cultures were longer and plumper. Infectivity of such cultures for mice was determined on 5 occasions; it varied between 10^{-7} to $10^{-8.5}$.

Vaccines were prepared from tissue culture material in the following manner: after discarding the condensation fluid from each flask, 5 cc of a diluent was added to each

Rowlands, I. W., Van den Ende, M., Bargman, H. E., Curtis, E. E., and Shepherd, M. A., *Lancet*, 1945, **2**, 734.

3 Plotz, H., Bennett, B. L., Reagan, R. L., Bell, E. J., Hamilton, H. L., and Snyder, M. J., Report to the Director, USA Typhus Commission, 23 September 1944.

4 a. Smadel, J. E., Rights, F. L., and Jackson, E. B., Reports to the Director, USA Typhus Commission, submitted on 2 October 1944 and 19 June 1945, respectively; b. Smadel, J. E., Rights, F. L., and Jackson, E. B., *J. Exp. Med.*, 1946, **83**, 133; c. Smadel, J. E., Rights, F. L., and Jackson, E. B., in press.

† Isolated by Dr. R. Lewthwaite in New Guinea and brought to the United States by the U. S. Navy.

5 Zinsser, H., Plotz, H., and Enders, J., *Science*, 1940, **91**, 51.

TABLE I.
Resistance of Vaccinated Mice to Scrub Typhus.

Vaccine No.	Dilution of challenge inoculum									Titer 50% lethal (log)	Immunity index (log)	M.L.D.
	10-1	10-2	10-3	10-4	10-5	10-6	10-7	10-8	10-9			
440802												
Controls			5/5	5/5	5/6	5/5	2/4	2/6		—7.2		
Vaccine			3/8	0/7	1/7	1/8				—3.0	4.2	15,800
440919												
Controls	10/10	10/10	10/10	9/10	10/10	10/10	10/10	1/9		—7.6		
Vaccine	10/10	6/10	4/10	6/10	3/10	3/10	1/10			—3.9	3.7	5,010
440928												
Controls		10/10	10/10	9/10	10/10	10/10	6/10	1/10		—7.2		
Vaccine		4/8	3/8	1/8	2/8	0/8	3/8			—3.0	4.2	15,800
441003												
Controls		10/10	10/10	10/10	10/10	10/10	5/10	2/10		—7.2		
Vaccine		6/10	4/10	3/10	2/10	3/10	1/10			—3.2	4	10,000
441009												
Controls		10/10	10/10	10/10	10/10	9/10	8/10	2/10		—7.4		
Vaccine		5/10	8/10	3/10	1/10	2/10	2/10			—3.5	3.9	7,950
441017												
Controls		10/10	10/10	10/10	10/10	10/10	7/10	9/10		—8.3		
Vaccine		5/6	7/10	2/9	5/10	6/10	2/8			—4.4	3.9	7,950
441024												
Controls		6/6	6/6	6/6	6/6	6/6	3/6	2/6		—7.3		
Vaccine		4/6	2/6	3/6	1/6	0/6	0/6			—2.9	4.4	25,000
110645												
Controls		10/10	10/10	10/10	10/10	10/10	9/9	7/10	7/9	—9.1		
Vaccine		7/7	9/10	6/10	1/10	2/10	0/10	4/10	0/10	—4.6	4.5	31,600

Denominator indicates size of group challenged.
Numerator indicates number of mice in each group that died from the challenge inoculation.

flask; this solution consisted of one part M/15 Sorenson's phosphate buffer pH 7.0 and 4 parts physiological saline solution, and contained merthiolate or formaldehyde as a preservative. The preservative was used at a final concentration of 1:10,000 of merthiolate or 0.2% of USP formaldehyde; a combination of both was employed in a few instances. The cells were scraped from the agar surface by means of a glass rod and pipetted into a 250 cc Pyrex bottle containing glass beads. The material was maintained at 4°C for 4 to 6 days and then frozen at — 20°C overnight. It was then thawed at room temperature and again frozen at — 20°C. The next morning it was thawed at room temperature and centrifuged in a horizontal centrifuge at 1,500 r.p.m. for 15 minutes. The supernatant fluid was removed and saved. The sediment, still in the original bottle, was shaken on a machine for one hour. The supernatant fluid which had been saved was added to the shaken material and the mixture was centrifuged in a horizontal machine at 1,000 r.p.m. for 10 minutes. The supernatant fluid, which represented the vaccine, was stored at + 4°C until the results of aerobic and anaerobic cultures and safety tests in mice became available.

The vaccines were tested in the following manner: groups of from 30 to 80 Swiss mice, weighing from 15 to 18 g, were inoculated with the materials under investigation. Each animal received 4 intraperitoneal injections of 0.25 cc amounts of vaccine at 5-day intervals; in some instances 2 intraperitoneal injections of 0.5 cc amounts of vaccine were given at a 7-day interval. Some of the vaccines were also used to immunize mice by the subcutaneous route. Twelve to 14 days following the last dose of vaccine treated mice were tested for resistance to infection with the homologous strain of scrub typhus. For this challenge inoculation serial 10-fold dilutions of fresh infectious yolk sac suspensions were prepared in a mixture containing physiological saline solution and 10% normal rabbit serum and placed in an ice bath until the inoculation was completed. Each mouse received an intraperitoneal injection

of 0.2 cc amounts of one of the dilutions of infectious material. An attempt was made to cover the entire range of dilutions from 10^{-2} to 10^{-7}. Normal control mice were inoculated with each dilution of the challenge material immediately after the test animals had received portions of the same suspension. Infected mice were observed for 21 days and deaths recorded. All deaths before the sixth day were considered as non-specific.

The resistance of vaccinated mice to infection with *R. orientalis* was estimated in the following manner. The 50% end-point method (Reed-Muench) of calculating infective titers was applied to the data obtained when groups of vaccinated and control mice were challenged with serial 10-fold dilutions of infectious material. In those experiments in which some mice survived among the group that received the most concentrated challenge material employed, it was assumed, for the purpose of calculating the end-point, that if a still more concentrated inoculum had been used then all of the mice would have succumbed.

Results. The results obtained in immunological tests on 8 scrub typhus vaccines are summarized in Table I. It is observed that under the conditions of these experiments, an immunity index of from 3.7 logs to 4.5 logs was found. In other words, these vaccines induced protection against from 5,000 to 32,000 MLD's of *R. orientalis*. A number of other vaccines were prepared which failed to show the relatively high immunity index displayed by those illustrated in Table I. These had indices of 1.1 to 2.43 logs (2.3 - 2.4 - 2.43 - 1.1 - 2.1 - 1.9 - 1.7 logs). It is felt that these poorer vaccines resulted from the fact that the culture flasks were not adequately examined so as to include in the vaccines only those cultures that were rich in *R. orientalis*.

The vaccines mentioned in Table I elicited a demonstrable resistance in mice when they were injected intraperitoneally and the animals were challenged by the same route. When certain of these potent vaccines were administered subcutaneously and the mice were challenged subsequently by the intraperitoneal route, the immunity indices varied from zero to 1.9 logs.

The immunological specificity of the resistance induced by scrub typhus vaccines was evident from control experiments. A normal chick tissue "vaccine" was prepared from agar tissue cultures in the same manner as was the scrub typhus vaccines except that infectious inoculum was omitted. The mice received 2 intraperitoneal injections of 0.5 cc at 7-day intervals and were challenged after 14 days. No protection was elicited in the treated mice; the infective titer was $10^{-7.2}$ in test and control animals. Likewise, a similar experiment was performed using a commercially prepared epidemic typhus vaccine. The immunity index was 1.8 logs or 63 MLD's protection. These data are summarized in Table II.

A series of experiments in which mice received "booster" injections of vaccine illustrate the value of this type of immunization procedure. A group of mice that had received the usual course of injections of vaccine 441009 was held for 30 days and then given a booster injection of 0.5 cc of the vaccine by the intraperitoneal route. The vaccine had an immunity index of 3.9 logs when tested by the usual method (Table I) but when the booster injection was employed, the index became 4.9 logs. Thus, the induced resistance increased from 7,950 to 80,000 MLD's. A similar set of comparative experiments was made with vaccine 441024. The immunity index was 4.4 logs when the ordinary assay was done but rose to 5.4 logs in the experiment in which the booster injection was used. Here again the protection was increased 10-fold, from 25,000 to 250,000 MLD's.

The salutary effect of booster injections is even better illustrated by the results obtained with a poor scrub typhus vaccine. Vaccine 441206, made with the Kostival strain and inactivated with 0.2% formalin, was injected intraperitoneally or subcutaneously into 2 groups of mice. 0.5 cc doses were given on 2 occasions 7 days apart. The animals were all held for 30 days when half of each group received a second injection of 0.5 cc of the same vaccine by the original route. Twelve days later all the mice were tested for re-

TABLE II.
Effect of Control Materials on Resistance to Scrub Typhus.

Vaccine	Dilution of challenge inoculum							Titer 50% lethal (log)	Immunity index (log)	M.L.D.
	10-2	10-3	10-4	10-5	10-6	10-7	10-8			
Normal chick tissue "vaccine"	10/10	10/10	10/10	9/10	6/9	9/10		—7.2+	0	0
Epidemic typhus vaccine	9/9	9/10	6/10	7/10	2/10	5/10		—5.4+	1.8	63
Controls	10/10	10/10	10/10	7/9	9/10	6/10	3/10	—7.2+		

TABLE III.
Effect of Booster Inoculation on Resistance to Scrub Typhus.

Vaccine No. 441206	Dilution of challenge inoculum							Titer 50% lethal (log)	Immunity index (log)	M.L.D.
	10-2	10-3	10-4	10-5	10-6	10-7	10-8			
I.P. 2 times	6/7	7/8	5/8	1/8	7/8	0/8		—4.7	1.8	63
I.P. 2 times + booster	6/7	1/7	3/7	1/8	0/8	0/7		—2.8	3.7	5,010
Unvaccinated controls	8/8	8/8	8/8	8/8	4/8	3/8	1/8	—6.5		

sistance to infection, the results are summarized in Table III. It is evident that the immunity index obtained when one course of vaccine was given intraperitoneally was 1.8 logs while the index obtained when a booster dose was given was 3.7 logs, almost a 100-fold increase. It is of interest to note that this vaccine, when administered subcutaneously, induced no resistance even when the booster technic was employed.

Discussion. The degree of protection afforded by the 3 types of scrub typhus vaccines[2,3,4] is comparable. The vaccines prepared by Fulton and Joyner[2] or Smadel, Rights and Jackson,[4] as well as ourselves, elicit immunity when the vaccines are injected intraperitoneally but none when given by the subcutaneous route. It is believed that the intraperitoneal immunity is specific, for control materials inoculated intraperitoneally do not induce such protection. The immunity index induced by the scrub typhus vaccines prepared from the lungs of cotton rats infected by the intranasal route showed an immunity index of 3.7 logs[2] while the vaccines prepared from the lungs of white rats, after intravenous inoculation, varied from 2.3 logs to 4.5 logs.[4] Vaccines were prepared from agar tissue cultures which afforded an immunity index of from 3.7 logs to 4.5 logs.

While the manufacture of large amounts of agar tissue culture scrub typhus vaccine would present certain technical difficulties, this method is feasible for making sufficient amounts to protect certain particularly exposed personnel. An advantage in the agar tissue vaccine over the others described is that a relatively clean product can be obtained. Of the various tissue culture methods employed in the preparation of rickettsial vaccines, the yolk sac method no doubt would be the best for large scale production. A successful vaccine has not as yet been prepared from this material, one reason being the deleterious effect of ether upon the antigen.

Summary. Inactivated vaccines prepared from agar tissue cultures infected with *R. orientalis* are capable of protecting mice against infection with scrub typhus.

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A Convenient Test Animal for the Detection of the Leukocytosis-Promoting Factor of Exudates.*

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Earlier studies have demonstrated in inflammatory exudates the presence of a factor liberated by injured cells and *per se* capable of reasonably explaining the mechanism of leukocytosis associated with numerous inflammatory processes.[1] This factor has been termed the leukocytosis-promoting factor (abbreviated as LPF). Chemically, the active material is associated with the pseudoglobulin fraction of exudates.[2,3] Besides being capable of discharging immature leukocytes into the circulating blood, the LPF induces a specific growth effect on granulocytes and megakaryocytes in the bone marrow.[4] The presence of the factor in the exudates of rabbits has been confirmed by Reifenstein.[5] These studies have also received confirmation in the hands of Page and his associates.[6]

The purpose of the present brief communication is to demonstrate that the guinea pig can also be conveniently utilized as a test animal for the presence of the leukocytosis-promoting factor in canine inflammatory exudates. Prior to this study, observations have been carried out exclusively on dogs. The finding of a smaller test animal for the assay of LPF would obviously be of distinct advantage for future studies.

Experimental. Exudative material was obtained from the pleural cavity of dogs by the intrapleural injection of turpentine, as previously described.[7]

The whole exudate, in quantities varying from 0.5 to 5 cc, was injected into the peritoneal cavity of guinea pigs following the determination of the basal white cell count. The blood sample was obtained by nicking the toe-pad with the sharp blade of a razor. The exudative material was injected intraperitoneally, for technical convenience, rather than intravascularly, as in previous studies. This method, however, entails certain disadvantages. Injection into the peritoneal cavity may be accompanied by the puncture of a visceral structure with consequent introduction of the material into either intestine or some other organ. Furthermore, white counts were made every hour subsequent to the injection of the exudative material for a period of about 4 hours. It was found that after a longer interval a frank peritonitis frequently developed, which in turn liberated its own LPF and thus confused the results. But by reducing the period of study to the first 4 hours following introduction of the material, this error could to a large extent be eliminated. Nevertheless, intraperitoneal injections obviously favor a less efficient absorption of material than direct intravascular injection. As a consequence, the final results obtained show definitely more individual variation than was encountered in dogs.

The results following intraperitoneal injection of canine exudative material are assembled on Table I. It is clear that at least in 4 out of 6 experiments the introduction of an exudate was followed by a definite increase in the number of circulating

* This represents No. 33 in a series entitled "Studies on Inflammation." This study was aided in part by a grant from the Duke University Research Fund.

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2 Menkin, V., *Arch. Path.*, 1940, **30**, 363.

3 Menkin, V., and Kadish, M. A., *Am. J. Med. Sci.*, 1943, **205**, 363.

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5 Reifenstein, G. H., Ferguson, J. H., and Weiskotten, H. G., *Am. J. Path.*, 1941, **17**, 233.

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7 Menkin, V., *Am. J. Path.*, 1934, **10**, 193.

TABLE I.
Effect of Exudative Material on Number of Circulating Leukocytes in the Guinea Pig.

Guinea pig No.	Amt of exudate inj.	Basal white blood cell level	Highest level of white blood cells attained within 4 hrs after inj. of exudate	Absolute increase in white blood cells
	cc	per mm³	per mm³	per mm³
Z	0.5	6,640	9,500	2,860
11	1	13,500	21,800	8,300
W	2	16,850	19,675	2,825
J	3	3,325	11,500	8,175
24-86	5	15,400	27,500	12,100
V	5	13,000	22,050	9,050
Avg		11,453	18,671	7,218

TABLE II.
Effect of the Leukocytosis-promoting Factor (LPF), Injected Intraperitoneally, on Leukocyte Level in the Guinea Pig.

Guinea pig No.	Amt. of LPF	Basal WBC level (per mm³)	Highest level attained within 4 hr after injec. (per mm³)	Absolute increase in WBC (per mm³)
X	3.0 cc	12,600	17,725	5,125
11	5.0 "	13,200	21,800	8,600
24-39	5.0 "	8,712	12,175	3,463
24-88	5.0 "	15,850	17,375	1,525
100	6.0 mg	4,638	7,150	2,512
9	6.0 "	8,500	15,750	7,250
24-36	8.0 "	3,600	11,275	7,675
17-60	8.5 "	12,750	18,400	5,650
24-87	11.0 "	12,727	30,575	17,848
24-47	12.0 "	9,075	14,550	5,475
11	13.0 "	17,225	58,975	41,750
24-36	22.0 "	7,725	15,625	7,900
Avg		10,550	20,115	9,565

leukocytes. The average increase in the number of circulating leukocytes is 7,218 or 63%. This increment in the white cells of guinea pigs is about of the same magnitude as encountered previously on dogs.[1] The actual effect throughout the duration of an experiment is illustrated in Fig. 1 (g.p. 24-86, Table I).

Having established the presence of the leukocytosis-promoting factor in dog exudate, which is apparently active on the number of circulating leukocytes in the guinea pig, observations were undertaken in an endeavor to determine whether the active principle, *i.e.*, the LPF itself is likewise active in the guinea pig. The factor was recovered from exudates, with only slight modifications, essentially as described by one of us in an earlier study.[8] The material dissolved in saline was injected in varying doses (Table II) into the peritoneal cavity of guinea pigs. In 9 out of 13 experiments, the LPF definitely increased the number of circulating leukocytes of the guinea pig. When, as explained above, the disadvantages attending intraperitoneal injections are taken into consideration, it is readily seen that 9 out of 13 experiments in which activity was demonstrated seem to be of definite significance. The average increase in the number of circulating white cells is 9,565 or 90.7%. The recovery of the LPF from the crude exudate, in which other factors are likewise present, readily explains the somewhat more pronounced effect obtained (cf. Table I and Table II). The course of an experiment with the leukocytosis-promoting factor (guinea pig 24-87) is illustrated in Fig. 1. A study was also undertaken to determine the normal range of variation in the white

[8] Menkin, V., *Arch. Path.*, 1945, **39**, 28.

TABLE III.
Variation in Number of White Cells in Blood of Normal Guinea Pig.

Guinea pig No.	Lowest No. of leukocytes per mm³ within about 5 hr	Highest No. of leukocytes per mm³ within about 5 hr	Absolute increase in white blood cells per mm³
11	16,025	17,950	1,925
24-32	8,525	13,400	4,875
24-96	6,805	9,180	2,375
100	5,050	7,975	2,925
24-39	7,850	9,725	1,875
24-47	7,375	13,825	6,450
24-36	6,300	7,275	975
Avg	8,276	11,333	3,057

TABLE IV.
Effect of Leukocytosis-promoting Factor (LPF), Injected Subcutaneously, on Leukocyte Level in the Guinea Pig.

Guinea pig No.	Amt. of LPF	Basal WBC level (per mm³)	Highest level attained within 4 hr after injec. (per mm³)	Absolute increase in WBC (per mm³)
2-22	40 mg	15,150	23,500	8,350
2-23	35 "	18,150	32,950	14,800
2-24	39 "	19,900	26,900	7,000
2-25	40 "	9,650	15,900	6,250
2-26	5 cc	12,100	22,000	9,900
a	10 "	12,350	22,950	10,600
2-28	10 "	11,950	14,600	2,650
2-31	10 "	12,100	28,450	16,350
2-29	10 "	10,050	23,950	13,900
b	5 "	8,000	18,700	10,700
17-58	42 mg	29,300	53,200	23,900
2-32	35 "	26,200	35,700	9,500
Avg		15,408	26,566	11,158

TABLE V.
Effect of Injections in Peritoneal Cavity and in Subcutaneous Tissue of the Guinea Pig of Various Materials Other than Exudate or LPF.

Guinea pig No.	Type and amt of injected material	Basal WBC level (per mm³)	Highest level attained within 4 hr after injec. (per mm³)	Absolute increase in WBC (per mm³)
	Intraperitoneal injec. cc			
20	1 liver extr. concentr. in barbiturate buffer	10,925	13,925	3,000
A	5 saline	12,850	15,600	2,750
24-36	2 human serum	5,775	8,750	2,975
B	2 canine serum	9,500	11,600	2,100
C	2 " "	11,100	12,700	1,600
D	2.5 broth	10,100	10,800	700
	Subcutaneous injection			
2-33	10 saline	10,550	13,900	3,350
2-34	5 "	9,800	14,350	4,550
2-38	5 "	15,700	20,700	5,000
2-35	5 necrosin	11,850	13,250	1,400
2-36	2 "	10,950	11,000	50
	Avg	10,827	13,325	2,498

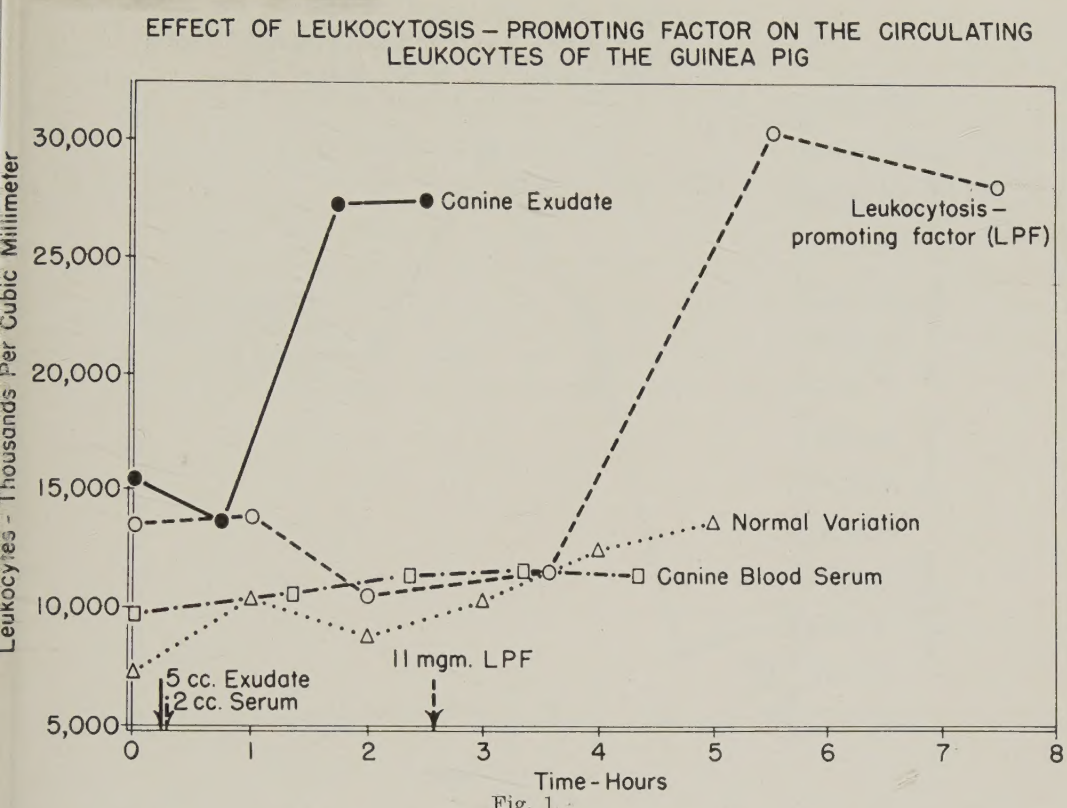

Fig. 1.

cell count during a period of about 5 hours in uninjected guinea pigs. The data are presented in Table III. (Fig. 1). Except for one animal in which there was an increase of 6,450 cells, the usual range of maximum rise in white cells definitely tended to be lower than in the injected guinea pigs. (Tables I, II and III). The average increase was 3,057 cells or 36.9%. It is quite clear from a survey of observations of this nature, in comparison with observations on experimental animals, (Tables I and II) that the leukocytosis-promoting factor obtained from a canine exudate is definitely active in inducing a leukocytosis in the guinea pig when the active substance is injected intraperitoneally.

Nevertheless, in view of the wide variations obtained from animal to animal when intraperitoneal injection of the active substance was employed, and in view, as explained above, of the reason for these fluctuations in results, a different route of injection was used in a series of experiments. The leukocytosis-promoting factor or LPF either in the fluid state or in dry form and dissolved in 5 to 10 cc of physiological saline was now injected subcutaneously. This was done, as a rule, in the region of the thigh. Such procedure induced a very rapid rise in the number of circulating leukocytes. Sometimes, as early as a half hour after introduction of the active material, an appreciable leukocytosis ensued. The period of an experiment extended, as a rule, for about 4 hours. During this interval a leukocytosis usually developed. The rise in the number of circulating leukocytes was found to be appreciable in 11 out of 12 experiments (Table IV). The average rise in 12 experiments was found to be 11,158 or a rise of 72.4%.† The course of one experiment is

† The fact that the average basal level in the series of guinea pigs listed in Table IV is somewhat higher than in the other series of experiments is largely explained by the fact that 2 of

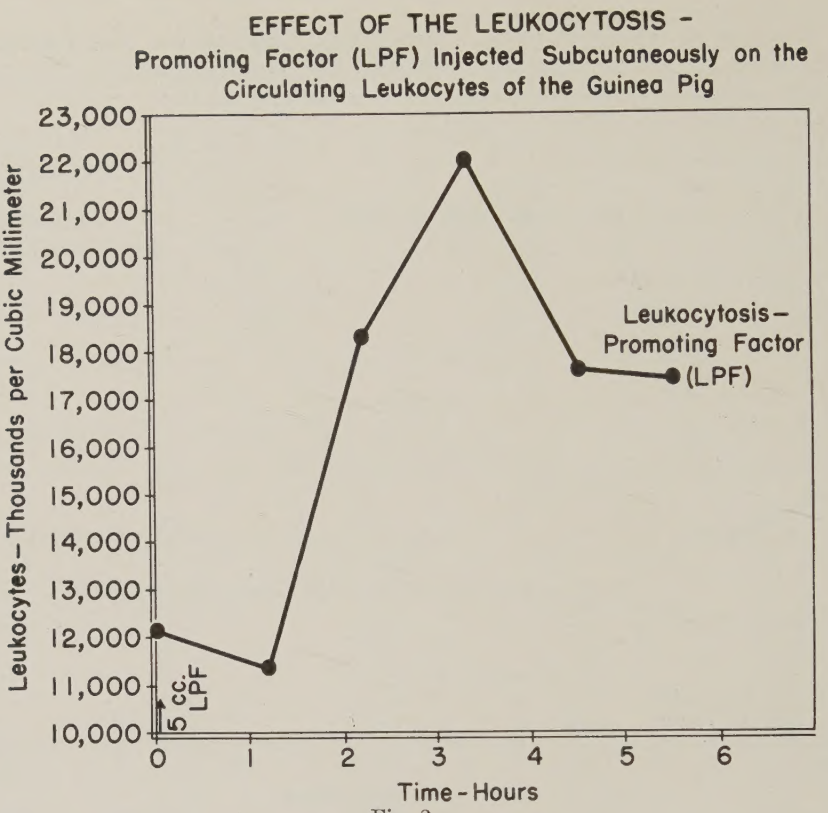

Fig. 2.

illustrated in Fig. 2. It is thus quite clear that the results are more constant and dependable when, in the guinea pig, the subcutaneous route of infection of the leukocytosis-promoting factor was employed.

Finally, as an additional control, various types of material unrelated to the leukocytosis-promoting factor of exudates have been injected into the peritoneal cavity and the subcutaneous tissue of guinea pigs. Those have included diversified substances such as liver extract, saline, broth, human and canine blood serum, and necrosin recovered in turn from exudates. The results of these experiments are assembled together in Table V (also Fig. 1). It is clear that, in contrast to the effect of the exudate and the leukocytosis-promoting factor, these materials have failed to alter appreciably the number of circulating leukocytes. The average maximum rise in the number of leukocytes is 2,498, or 23%. It is to be noted that these non-specific substances were all injected as solutions, precisely as in the case of the exudative material or the LPF. This is important, for when an insoluble particulate material is injected, for instance, in the peritoneal cavity, a peritonitis is prone to develop early; and this may in turn confuse the final interpretation of results.

Conclusions. An exudate obtained from a dog, which contains the leukocytosis-promoting factor, induces a leukocytosis when injected into the peritoneal cavity of a guinea pig. Similar results are obtained with the recovered leukocytosis-promoting factor of exudates injected either intraperitoneally or subcutaneously. The effect is quite specific. It fails to occur when other types of soluble materials are utilized. Consequently, the guinea pig presents itself as a suitable test animal for detecting the presence of the leukocytosis-promoting factor in inflammatory

the animals already had a leukocytosis prior to the injection of the material. (Guinea pigs Nos. 17-58 and 2-32, Table IV).

exudates, particularly if the material is injected into the subcutaneous tissue.

Finally, as pointed out frequently in the past, these studies may have clinical application, for it is well known that the prognosis of an infectious process is to a large extent referable to the number of circulating leukocytes. For this reason studies have been initiated to determine the effectiveness of the leukocytosis-promoting factor on man. These studies are now in progress and will form the subject of a separate future communication *in extenso*. It can be pointed out, however, that 8 different patients with normal white cell counts have been injected intravenously with doses ranging from about 18 to 230 mg of active canine LPF. Within a few hours the white blood cell count increased without any change in temperature, and within several hours the count had risen 80 to 150%, showing that the material is both innocuous and potent on human beings. These studies are being carried out by 2 of us (V. M. and E. U.) in collaboration with Dr. E. G. Goodman of the Department of Medicine.